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(71) Applicant: UNIVERSITY OF WASHINGTON [US/US]; Office of Technology Transfer, Suite 200, 1107 N.E. 45th Street, Seattle, WA 98105 (US).

(72) Inventors: BOYLE, Edward, M.; 6036 29th Avenue N.E., Seattle, WA 98115 (US). KOVACICH, John, C.; 4756 University Village Place N.E. #270, Seattle, WA 98105 (US). NORBERT, Frank; Gutleuthofweg 14, D–69118 Heidelberg (DE). CANTY, Timothy, O., Jr.; 2418 E. Miller Street, Seattle, WA 98112 (US). MORGAN, Elizabeth; 2570 5th Avenue W., Seattle, WA 98119 (US). POHLMAN, Timothy, H.; 8240 Fairway Drive, N.E., Seattle, WA 98115 (US). VERRIER, Edward, D.; 1018 36th Avenue E., Seattle, WA 98112 (US).

(74) Agents: PARMELEE, Steven, W. et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 98411 (US).

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(54) Title: INHIBITION OF NF–κB MEDIATED TISSUE INJURY USING DITHIOCARBAMATE DERIVATIVES

(57) Abstract

Methods for treating inflammatory responses in a mammalian patient based on administration of cyclic dithiocarbamate (DTC) and thiuramsulfide (TS) derivatives are disclosed. Antiinflammatory effective amounts of cyclic DTC or TS derivatives are administered to a mammalian patient to treat ischemia/reperfusion injury, systemic inflammatory response syndrome (SIRS) and oxidative stress–related conditions. Useful compounds include DTCs and TSs having one or more structural modifications selected from substituent(s) in a bridge portion of a ring member of the compound, heteroatom(s) within the bridge portion, carbonyl function(s), and/or dehydrogenation to yield one or two double bonds within the compound. Methods of treatment specifically inhibit NF–κB activity and oxidative stress effects while not substantially inhibiting beneficial pathways of inflammation.

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INHIBITION OF NF-κB MEDIATED TISSUE INJURY USING DITHIOCARBAMATE DERIVATIVES

Background of the Invention

Oxidative stress and other inflammatory responses play a prominent role in the pathophysiology of acute and chronic cardiovascular disease, shock and sepsis, ischemia-reperfusion injury, aging, cancer and AIDS. There is increasing evidence that a major component of these inflammatory responses are attributable to changes within the microvasculature, especially at the level of the vascular endothelium.

The microvasculature actively participates in maintaining normal cardiovascular homeostasis by controlling membrane permeability, vasomotor tone, coagulation and inflammation. Verrier et al., Ann. Thorac. Surg. 62(3): 915-22, 1996. This is done by tightly regulating the expression of endothelial-derived biologically active agents, in the form of either surface proteins or locally secreted soluble factors, that exert opposing effects to either increase or decrease the degree of vasoconstriction, promote or prevent coagulation, enhance or impede platelet and leukocyte adhesion Verrier et al., Ann. Thorac. Surg. 62(3): 915-22, 1996. In the quiescent state these dynamic endothelial cell functions work in concert to promote blood flow.

However, in response to injury, such as hypoxia, exposure to cytokines, endotoxin, cholesterol, nicotine, or physical injury in the form of surgical manipulation or hemodynamic shear stress, endothelial cells undergo phenotypic changes that allow them to participate actively in the inflammatory response. This response, termed endothelial cell activation, enhances vasoconstriction, coagulation and leukocyte adhesion. Pober et al., Transplantation 50(4):537-44, 1990; Pober, Warner-Lambert/Parke-Davis award lecture. Am. J. Pathol. 133(3):426-33, 1988.

Although these vasomotor, procoagulant and proinflammatory changes likely exist as protective mechanisms, when the stimuli are severe as in the case of cardiovascular disease, shock and sepsis, or chronic, as occurs with atherosclerosis or aging, the response may become excessive resulting in damaged tissue and impaired organ function.

At present the only therapy potentially available to inhibit tissue injury related to endothelial cell activation is immunosupression with steroids or anti-neutrophil integrin monoclonal antibodies, neither of which have achieved widespread clinical application. Johnson et al., Clinical Immunother. 3:8-14, 1995; Sharar et al., Surgery 110(2): 213-9, discussion 219-20, 1991. Moreover, these approaches attempt to block endothelial cell activation after it occurs, whereas a more desirable approach is to prevent activation from occurring altogether.

Clinically accepted techniques for preventing microvascular activation induced organ injury clinically have not yet been determined. Current approaches under development focus on inhibition of single components of the overall inflammatory response, mostly "down stream" components (i.e., which exert their effects after inflammation has been initiated). Proposed inhibitory agents in this context include monoclonal antibodies and small molecule inhibitors to prevent neutrophil-endothelial cell interactions, inhibitors of oxygen derived free radicals and protease inhibitors to prevent neutrophil mediated injury after neutrophils become adherent, and inhibitors of neutrophil chemotaxis which target such molecules as C5a, PAF, or LTB4.

Among the better known agents which have been sought to modulate inflammatory responses are monoclonal antibodies, particularly antibodies that target adhesion molecules expressed on cytokine activated endothelium. Gillinov et al., Ann. Thorac. Surg. 57(1):126-33, 1994). There is presently an increasing number of candidate monoclonal antibodies available, including ones that block neutrophil based adhesion molecules before neutrophils can adhere. Verrier et al., J. Card. Surg.

8:309-12, 1993. However, activated endothelial cells express a variety of important adhesion proteins, at different time intervals. Thus, a mixture of these blocking agents administered in a complex protocol would likely be required to block the full course of endothelial cell activation.

Additional monoclonal antibodies that bind to selectins (eg., E-selectin or P-selectin), as well as circulating oligosaccharide antagonists that block interactions between neutrophil ligands and endothelial selectins, have been shown to prevent rolling and subsequent adherence of neutrophils. These agents have all been reported to inhibit ischemia-reperfusion or septic shock in various animal models. Phillips et al., J. Clin. Invest. 96:2898-2906, 1995; Bucky et al., Plast. Reconstr. Surg. 93(7):1473-80, 1994; Doerschuk et al., J. Immunol. 144(6):2327-33, 1990; Harlan, Blood 65(3):513-25, 1985; Winn et al., Agents Actions Suppl. 41:113-26, 1993. However, blocking neutrophil-endothelial cell adhesion at this level may substantially increase susceptibility to infection, limiting this approach as a therapeutic strategy. Sharar et al., Surgery 110:213-9, 1991. This is consistent with what is known about patients having congenital defects in neutrophil adhesion molecules, who generally die early from severe infection. Phillips et al., J. Clin. Invest. 96:2898-2906, 1995.

Numerous additional obstacles have been encountered in using monoclonal antibodies as therapeutic agents clinically, including those aimed at blocking neutrophil adhesion molecules. For example, many patients exhibit acute allergic reactions to these antibodies, which can result in clinical manifestations ranging from itching and edema to anaphylactic shock. In addition, some patients develop antibodies to foreign proteins found in monoclonal antibodies, resulting in anaphylaxis if the antibody is administered repeatedly. This can limit the efficacy these drugs to one or two uses. Furthermore, monoclonal antibodies are costly and difficult to make and distribute, particularly in large quantities.

A more fundamental problem with the foregoing approaches is that they generally target single, downstream events in the inflammatory pathway. However, inflammation is generally initiated and amplified by redundant, overlapping pathways. In many cases, if an agent is successful in knocking out one pathway, other pathways may remain intact to promote tissue injury. Ideally, inhibition of inflammatory responses will target upstream regulatory mechanisms to prevent endothelial activation before it occurs, rather than trying to interrupt downstream mechanisms such as neutrophil-mediated inflammation.

Transcriptional regulation of inflammatory genes activated by oxidative stress provides a potential focal point for controlling endothelial cell activation. In particular, attention is increasingly focused on the signal transduction pathways, including the various protein kinase cascades, thought to be involved in entothelial activation.

Quiescent endothelial cells lining the microvasculature respond to injury by activating signal transduction pathways that transmit signals through the cytoplasm to the nucleus. These signals result in the transcription, translation, and expression of proinflammatory, procoagulant, and vasoactive genes that characterize endothelial cell activation. Davies et al., Ann. Surg. 218:593-609, 1993; Verrier et al., Ann. Thorac. Surg. 62: 915-22, 1996. Additional "downstream" events involved in endothelial cell activation include microthrombosis from tissue factor expression, neutrophil adhesion secondary to expression of leukocyte adhesion molecules (eg., E-selectin, VCAM-1, ICAM-1, P-selectin), and the release of cytokines, chemokines and growth factors (IL-1, IL-6, IL-8, b-IFN, MCP-1, TNF, GM-CSF, M-CSF, G-CSF) that contribute to local cellular activation and chemotaxis.

Deletional and mutational analyses of genes associated with downstream entothelial activation mechanisms suggest that many such genes are transcriptionally regulated by a common transcription factor, nuclear factor-kappa-B (NF-κB).

Baeuerle et al., Ann. Rev. Immunol. 12:141-79, 1994; Collins T, et al., Faseb. J. 9(10):899-909, 1995; Parry et al., Arterioscler. Thromb. Vasc. Bio. 15:612-621, 1995. NF-κB is composed of similar subunits as are found in other members of the "NF-κB/Rel family" of transcription factors. Baeuerle et al., Cell 87(1):13-20, 1996. Five distinct DNA-binding proteins in this family, p50, p52, p65 (also known as RelA), c-Rel, and RelB, are known to be involved in mammalian transcriptional regulation. Members of this family are defined by the presence of a highly conserved region of approximately 300 amino acids called the "rel homology domain", which bears the DNA binding site. A consensus DNA binding sequence is present, and the carboxy-terminus of the rel homology domain in all family members contains a highly specific cluster of positively charged amino acids that function as nuclear localization signals. The p65 subunit, in association with p50 or c-Rel (both present in NF-κB), is the most common heterodimer, subserving the trancriptional activation of many genes involved in immunologic and inflammatory responses. Parry et al., J. Bio. Chem. 269:20823-20825, 1994.

Because NF-κB is thought to regulate transcription of many genes involved in endothelial activation and is itself activated by diverse stimuli (see, eg., Baeuerle et al., Ann. Rev. Immunol. 12:141-79, 1994), this transciptional regulatory factor represents a potential target for blocking a range of inflammatory processes. Furthermore, because NF-κB also plays a regulatory role in oncogenesis and in replication of the human immunodeficiency virus (HIV), pharmacologic targeting of NF-κB will likely yield beneficial treatments for these disease states as well.

One method for modulating NF-κB activity *in vivo* has been proposed based on a gene therapy directed approach. Although gene therapy is commonly used to insert genes into patients with inherited gene defects, the concept of gene therapy is rapidly being broadened to include transient gene therapy to prevent and treat inflammatory responses. Brigham et al., Prog. Clin. Biol. Res. 361-5, 1994. In particular,

investigators have proposed a number of gene therapy techniques to inhibit NF-κB. These include anti-sense oligonucleotides directed at p65. However, gene therapy for a majority of inflammatory disorders attributable to endothelial cell activation is not currently practical, because methods to deliver genes and achieve high transduction or transfection efficiency are extremely limited.

Thus, a pharmacologic approach to inhibit NF-κB, would have significant advantages over gene therapy oriented therapies. In particular, a pharmacologic approach would likely be easier and safer than inserting foreign DNA into patients using viral or lipid based vectors, both of which may yield an inflammatory response by themselves. Furthermore, a successful pharmacologic agent might be amenable to repeated use to allow chronic treatment, in contrast with monoclonal antibody or gene directed approaches.

Although it has been demonstrated that NF-κB can be inhibited by a number of drugs *in vitro*, a pharmacologic agent that is non-toxic and effective for inhibiting inflammation has not heretofore been available for use in whole animal models. Among the diverse classes of compounds which have been explored for their potential to inhibit NF-κB activation and other inflammatory responses are the dithiocarboxylates, which include dithocarbamates (DTCs) and thiuramsulfides (TSs). There are at least 3 different dithiocarbamates that have been used clinically and experimentally. These include disulfiram (PTDS), diethyldithiocarbamate (DDT), and pyrrolidinedithiocarbamate (PDTC), as described for example in U.S. Patent No. 5,380,747 issued to Medford et al., on January 10, 1995.

Among this group of emerging experimental and drug compounds, DDT has been shown to protect against hepatotoxic agents and to exert anticarcinogenic activity. DDT has been used as an antitoxic agent to ameliorate the side effects of some chemotherapeutic agents. DDT has been of interest, not only for inducing intolerance of alcohol by increase of acetaldehyde, but also in the treatment of hereditary copper

thesaurismosis (Wilson's disease) and as an antidote against nickel carbonyl intoxication. Intravenous DDT has also been investigated for potential therapeutic use against HIV-related disease.

In the context of experimental antiinflammatory drugs, PDTC has been reported by some researchers to be an effective inhibitor of NF-κB *in vitro*. Kawai et al., J. Immunol. 154(5):2333-41, 1995; Schreck et al., J. Exp. Med. 175(5):1181-94, 1992; Collins et al., Faseb J. 9(10):899-909, 1995; Marui et al., J. Clin. Invest. 92(4):1866-74, 1993; Ziegler-Heitbrock et al., J. Immunol. 151(12):6986-6993, 1993. These reports, however, conflict with other data suggesting that PDTC exerts its effects posttranscriptionally, and therefore acts independent of NF-κB binding activity *in vitro*. Brisseau et al., Blood 85(4):1025-35, 1995.

In vitro expression of the adhesion molecules ICAM and VCAM is known to be NF-κB dependent. Collins et al., Faseb. J. 9(10):899-909, 1995. Thus, if PDTC were exerting its effects via inhibition of NF-κB, then these adhesion molecules should be downregulated following PDTC treatment, and PDTC treatment should prevent induction and upregulation of these adhesion molecules. In concordance with this hypothesis it has been shown that VCAM is downregulated in response to PDTC in human endothelial cells. Marui et al., J. Clin. Invest. 92(4):1866-74, 1993. However, militating against this hypothesis is the observation that ICAM-1 is not downregulated in human endothelial cells in response to free radicals, despite PDTC treatment. Weber et al., Arterioscler. Thromb. 14:1665-1673, 1994.

Other reports relating to PDTC activity have indicated that PDTC inhibits human fibroblast ICAM-1 expression in response to cytokines. Kawai et al., J. Immunol. 154(5):2333-41, 1995. PDTC has actually been shown to induce the transcriptional upregulation and surface expression of ICAM-1 in human endothelial cells. Munoz et al., Immunology 157: 3587-3597, 1996. *In vitro* data from human monocytes and promyelocytic cells has also yielded contrasting results

concerning the mechanism of action of PDTC. In addition, PDTC has been reported to potentiate LPS-induced TNF-α gene expression in murine macrophages (Brisseau et al., Blood 85(4):1025-35, 1995), but attenuates this activity in human monocytes. Ziegler-Heitbrock et al., J. Immunol. 151(12):6986-6993, 1993. TNF-α is also known to require NF-κB activation for its expression *in vitro*. Shakhov et al., J. Exp. Med. 171(1):35-47, 1990. Furthermore, PDTC has been reported to paradoxically have a stimulatory effect on activation of the transcription factor AP-1 which is also known to be important for the expression of several inflammatory mediators *in vitro*. Brisseau et al., Blood 85(4):1025-35, 1995; Meyer et al., Chem. Biol. Interact. 91(2-3):91-100, 1994.

Reported data regarding *in vivo* activity of PDTC is also conflicting. Studies by Lui have suggested that PDTC decreases NF-κB binding in response to LPS in a murine model of sepsis. Lui et al., J. Immunol. 159:3976-3983, 1997. However, Nathens and colleagues have recently reported that PDTC has no effect on LPS-induced NF-κB binding activity, or on mRNA levels of the proinflammatory molecules TNF and ICAM-1 induced *in vivo* by LPS in a rodent model. Nathens et al., Am. J. Respir. Cell Mol. Biol. 17:608-616, 1997.

With regard to possible antiinflammatory activity of PDTC, Liu and colleagues have reported that PDTC inhibited NF-κB activation in correlation with iNOS expression in a rat model of septic shock. Lui et al., J. Immunol. 159:3976-3983, 1997. They also suggest that PDTC treatment may prevent LPS-induced hypotension in the rat model.

However, whereas the rabbit model is widely accepted as a model system that is well correlated with human inflammatory intervention, the rat is far from an ideal study organism for this purpose. Many of the hemodynamic changes associated with the systemic inflammatory response syndrome and other dysregulated inflammatory responses in humans are observed in rabbits, but the same is not true in rats. Rats require exorbitantly large doses of endotoxins or cytokines to produce correlative inflammatory responses. This suggests that

fundamental interspecies differences exist between the immune system of rats compared to that of humans, which precludes drawing satisfactory correlations to humans from observations in rats. The rabbit on the other hand is well suited for studying responses to inflammatory mediators such as LPS, IL-1 and for evaluating inhibitory agents of inflammatory pathways and mechanisms. The rabbit and humans have similar dose responses to intravenously injected cytokines under both normal and experimental (eg., anesthetic) conditions. Okusawa et al., J. Clin. Invest. 81:1162-72, 1988; Wakabayashi et al., J. Clin. Invest. 87:1925-35, 1991; Wolff, J. Infect. Dis. 128:259-70, 1973.

In addition to these shortcomings, other *in vivo* studies have reported that PDTC does not inhibit NF-κB activation in a rat model of LPS induced tissue damage, as noted above. Nathens et al., Am. J. Respir. Cell Mol. Biol. 17:608-616, 1997. These reports collectively suggest that PDTC exerts its action without affecting NF-κB-mediated gene activation. Further, as Liu and coworkers point out in their discussion, PDTC caused a 85% inhibition of NF-κB activation but only a 48% reduction in iNOS mRNA expression and a 66% inhibition of iNOS activity.

In vitro studies have further indicated that activation of solely NF-κB is required for LPS and cytokine induced expression of iNOS. The results reported by Liu's group are not fully consistent with these findings, as NF-κB was clearly not the sole transcriptional regulator of LPS-induced gene expression in their model. Strict analysis of the reported blood pressure data is limited by the fact that only 2 time points are reported, thus trends over time could not be shown. Additionally, no other hemodynamic parameters were measured making it impossible to state precisely the mechanism by which PDTC might have inhibited LPS-induced hypotension. For instance, the reported effects may have been the result of an ionotropic or chronotropic effects on the heart, as opposed to an indirect effect on the peripheral vasculature as Liu's group proposed. It is therefore quite possible that PDTC

facilitated changes in blood pressure independently of gene activation.

Despite the reported advantages for dithiocarbamates, toxic side effects have been attributed to many representative compounds. These side effects include neurogenic effects and gastrointestinal ulcers. DDT is neurotoxic, in part, due to its reactivity with sulfhydryl groups, which impairs the antioxidant activity of glutathione and the DDT-inhibited detoxification of cytotoxic products of peroxidized lipids. An important function in the DDTC-induced neurotoxcicity is likely its ability to enhance preferentially the redistribution of endogenous copper to the brain, which can yield toxic side effects. Cytosolic and mitochondrial aldehyde dehydrongenase may also be involved in toxic side effects of certain DTCs.

Although the role of DTCs in mediating NF-κB activity remains unclear, the doses of PDTC required to achieve reported inhibition of NF-κB activation range from about 50 mg/kg to 200 mg/kg. Thus, reported dosages of this DTC compound necessary to achieve reported inactivation of NF-κB would be dangerously high in rabbits, and therefore likely in humans as well. In fact, data provided herein indicate that significant toxic effects from PDTC appear at doses as low as 15 mg/kg.

Recent studies by Frank and colleagues have studied carboxylated derivatives of DTCs that would be expected to be more hydrophilic than DTCs lacking an added carboxy group. Frank et al., Carcinogenesis 11:199-203, 1990; Hadjiolov et al., J. Cancer. Res. Clin. Oncol. 118:401-404, 1992; Frank et al., Toxicology 95:113-122, 1995. In particular, Frank and coworkers investigated the carboxylated derivatives, proline dithiocarbamate (ProDTC) and sarcosinedithiocarbamate (SDTC), which were synthesized by reacting proline or sarcosine with CS2 in alkaline milieu. These compounds were found to be less lipophilic than DDT, and, unlike DDT, do not enter the enterohepatic circle or penetrate the blood brain barrier. In

addition, Frank and coworkers found that ProDTC was more stable *in vivo*.

Investigating the comparative pharmacokinetics of DDT, ProDTC and SDTC in rats, Frank and colleagues tested the bioavailability, distribution of compounds in the organism, oxidation of the compounds to thiuramsulfides, cleavage to CS2, excretion in bile and urine. These studies showed substantial differences in pharmacokinetic behavior among the three compounds. The more toxic DDT had a short *in vivo* half-life, was oxidized to tetraethylthiuramdisulfide in blood, and was metabolized to high yields of CS2 in 24 hours. In contrast, ProDTC was more stable *in vivo*, was predominantly found in the urinary tract, and was excreted in urine. *In vivo* oxidation to corresponding thiuramdisulfides was also different for DDT, SDTC and ProDTC. Whereas DDT was found to be oxidized *in vivo* in only marginal amounts, ProDTC and SDTC were oxidized markedly. However, the corresponding thiuramdisufides were found predominantly in the blood in the case of SDTC, and in the urine in the case of ProDTC.

The differences in pharmacokinetics between DTC, ProDTC, and SDTC remain unexplained. These differences cannot be entirely explained by the added carboxy group in ProDTC and SDTC, because SDTC behaved comparably to DDT. It is possible that these differences to other characteristics of the compounds, for example the unique ring structure of ProDTC. A different redox potential may result from the different structures, which could be responsible for a different rate of oxidation to either TDS or to mixed disulfides. However, further studies are required to pinpoint the structural and functional attributes that mediate toxic effects, stability characteristics, clearance and other pharmacokinetic behaviors of DTCs and their derivatives.

In view of the foregoing, the roles and activities of DTCs and their derivatives in the context of mediating inflammatory changes remains largely uncertain. This uncertainty is consistent with the general understanding that dithocarbamates "exhibit extraordinarily complex chemical and

biological properties." Irons et al., EMBO J. 19:539-542, 1998.

It is therefore an object of the invention to develop methods to mediate inflammatory responses in mammalian patients at risk for developing adverse sequelae from such responses, which methods target inhibition of primary inflammatory mechanisms and pathways.

It is a further object of the invention to achieve the foregoing object in methods that employ a pharmacologic approach, so as to provide significant advantages over other approaches such as antibody-mediated inhibition and gene therapy. Consistent with this object, ancillary objects of the invention are to provide antiinflammatory methods which do not elicit adverse responses, such as adverse cytokine and cellular immune responses and secondary inflammatory responses, and which are permissive of chronic treatments.

Yet another object of the invention is to achieve a pharmacologic antiinflammatory treatment that employs highly efficacious and minimally toxic agents to optimize treatment and reduce patient morbidity.

Within the foregoing objects, the invention aspires to target primary inflammatory mechanisms and pathways. Thus, the invention seeks to resolve outstanding difficulties in disabling NF-κB-mediated inflammatory pathways, and in curtailing global impacts of oxidative stress-induced inflammatory responses. At the same time, a more challenging of objective of the invention is achieve focal inhibition of inflammatory pathways while avoiding disablement of beneficial inflammatory mechanisms, such as sustained cytokine function and immune competence that correlate with patient recovery.

Summary of the Invention

The invention fulfills these objects and satisfies other objects and advantages by providing methods for treating inflammatory responses in a mammalian patient based on administration of cyclic dithiocarboxylate (DTC and TS) derivatives.

Preferred methods within the invention involve administering an antiinflammatory effective amount of a cyclic DTC or TS derivative to a mammalian patient to yield a detectable or substantial antiinflammatory response (ameliorative or preventative) in the patient.

Cyclic DTC or TS derivatives for use within the invention exhibit antiinflammatory activity against a wide range of inflammatory conditions and disease states. These compounds comprise a discrete family of DTC and TS derivatives, and are preferably synthesized using a cyclic, secondary amine. Cyclic DTCs for use within the invention preferably share the general formula R1,R2-N-C(S)S-R3, wherein R1 and R2 constitute a cycle including the nitrogen atom of the dithiocarbamate. Preferred TS derivatives share the general formula R1,R2-N-C(S)-Sx-C(S)-N-R3,R4, wherein x is 1 to 8, and wherein R1, R2 and R3, R4 constitute independently a cycle including the nitrogen atoms of the respective dithiocarbamate residue.

Alternative embodiments of the invention employ a cyclic, substituted DTC or TS derivative, or a DTC or TS derivative having one or more structural modifications selected from substituent(s) in a bridge portion of a ring member of the compound, heteroatom(s) within the bridge portion, carbonyl function(s), and/or dehydrogenation to yield one or two double bonds within the compound. A further desired property of useful, cyclic DTC or TS derivatives within the invention is increased hydrophilicity as compared to that of a parent compound lacking the foregoing substituent(s), heteroatom(s), carbonyl function(s), and/or dehydrogenation.

In more detailed aspects of the invention, methods are provided to specifically inhibit NF-κB activity and/or oxidative stress effects while not substantially inhibiting other, beneficial pathways involved in inflammation. In yet other aspects, specific methods for inhibiting inflammatory responses without substantially elevating inflammatory-stimulating chemokines (eg., IL-8, MCP-1, and GRO) are provided.

Brief Description of the Drawings

Figures 1A and 1B depict examples of cyclic, secondary amine precursors for synthesis of cyclic derivatives of DTCs and TSs for use within the invention are provided in Figures 1A and 1B.

Figure 2 depicts examples of preferred substituents that may be with a cyclic, secondary amines to yield preferred, cyclic, substituted secondary amine precursors for synthesis of DTC and TS derivatives useful within the invention.

Figures 3 and 4 depict examples of cyclic, substituted secondary amines for synthesis of preferred DTC and TS derivatives for use within the invention.

Figure 5 depicts an exemplary synthetic reaction of CS_2 and L-proline in ethanol and NH_3 to yield a salt of a preferred DTC derivative, prolinedithiocarbamate (ProDTC) for use within the invention.

Figure 6 illustrates additional examples of reactions yielding preferred DTC derivatives for use within the methods of the invention.

Figures 7 and 8 depict hemodynamic parameters from rabbits treated with PBS + saline (closed squares), PBS + LPS (open diamonds), or PDTC + LPS (open circles). Figure 7 depicts Mean arterial pressure (MAP; mm Hg), and Figure 8 depicts Heart Rate (HR; beats/min). Data are presented as the mean for six rabbits in each group. The (*) symbol indicates values significantly different when compared to time 0 ($P < 0.05$ by paired t-test). Significantly different when compared to the saline control group, $P < 0.005$ (!) and $P < 0.05$ (#) by Fisher's least significance difference.

Figures 9-12 depict hemodynamic parameters from rabbits in Group 1 (PBS + saline; open squares), Group 2 (PBS + IL-1a; closed squares), Group 3 (PDTC + IL-1a; open circles), and Group 4 (ProDTC + IL-1a; closed circles). Percent change of the value at t = 0 min for Mean arterial blood pressure (MAP) (Figure 9); Systemic vascular resistance (SVR) (Figure 10); Cardiac output (CO) (Figure 11) and heart rate (HR) (Figure 12). Data are presented as the mean for six rabbits in

each group. (*) = Significantly different when compared to t = 0 by paired t-test analysis. Significant difference versus group 1 (!); group 2 (@); group 3 ($); or group 4 (#) ($P < 0.05$; by Fisher's least significance difference).

Figures 13-15 depict acid base status from rabbits in Group 1 (PBS + saline; open squares), Group 2 (PBS + IL-1a; closed squares), Group 3 (PDTC + IL-1a; open circles), and Group 4 (ProDTC + IL-1a; closed circles). Percent of the initial baseline value is shown for arterial pH (Figure 13), calculated base deficit (Figure 14), and arterial bicarbonate (HCO_3^-) (Figure 15). Data are presented as the mean for six rabbits in each group. Significant difference versus group 1 (!); group 2 (@); group 3 ($); or group 4 (#) ($P < 0.05$; by Fisher's least significance difference.

Figures 16-18 document rabbit chemokine levels in citrated plasma samples. Measurements of chemokine protein (ng/mL) by immunoassay are shown for IL-8 (Figure 16), MCP-1 (Figure 17), and GRO (Figure 18) in citrated plasma samples from rabbits treated as indicated in the Figures.

Figure 19, panel A represents HUVECs exposed to TNF-α (100U/ml) for a designated time period. The top panel is a Western blot using anti-IκBα antibody of HUVEC cytoplasmic extracts. The bottom panel is an electrophoretic mobility gel shift assay (EMSA) utilizing a P^{32} radiolabled oligo containing the NF-κB consensus sequence. These results show that NF-kB activity is increased in the nucleus as IκB disappears from the cytoplasm in response to TNF stimulation. Panel B represents HUVECs exposed to 2 hours hypoxia (2% Oxygen; labeled 0 minutes) followed by a designated time of reoxygenation. Again, the top panel is a Western blot with an anti-IκBα antibody and the bottom panel is an EMSA with the NF-κB consensus oligo. These results indicate that NF-κB is activated and translocates to the nucleus in response to hypoxia and reoxygenation in the absence of any degradation of IkBα from the cytoplasm.

Figure 20, panel A represents HUVECs exposed to H_2O_2 (500 μM) for a designated time course. Panel B represents

HUVECs exposed to pervanadate (PV) (100μM) for a designated time course. The top panel in each are Western blot using an anti-IκBα antibody and the bottom panels are EMSA's with the NF-κB consensus oligo. These results indicate that NF-κB is activated and translocates to the nucleaus in response to oxidative injury in the form of H_2O_2 or PV in the absence of any degradation of IκBα from the cytoplasm.

Figure 21, panel A provide a representative EMSA of nuclear proteins isolated from HUVECs exposed to TNF-α (100 U/ml) for 180 minutes with or without 60 minutes of pretreatment with various concentrations of ProDTC. The lanes are 1 to 6 from left to right. Lane 1 represents control cells that were exposed to saline only. Lane 2 represents the positive control cells that were exposed to TNF in the absence of ProDTC. Lanes 3-5 represent cells pretreated with 100, 200, or 500 μM of ProDTC respectively. Lane 6 is a cold competition assay of nuclear proteins incubated with cold probe prior to the addition of radiolabeled probe and successful elimination of the band demonstrates the NF-κB specificity. These data indicate that ProDTC has no effect on NF-κB binding activity induced by TNF.

Figure 21, panel B is a representative EMSA of nuclear proteins isolated from HUVECs exposed to pervanadate (PV) (200 μM) for 120 minutes with or without 60 minutes of pretreatment with various concentrations of ProDTC. The lanes are 1 to 6 from left to right. Lane 1 represents the control cells that were exposed to saline only. Lane 2 represents the positive control cells that were exposed to PV in the absence of ProDTC. Lanes 3-5 represent cells pretreated with 100, 200, or 500 uM of ProDTC respectively. Lane 6 is a cold competition assay of nuclear proteins incubated with cold probe prior to the addition of radiolabeled probe and successful elimination of the band demonstrates the NF-κB specificity. These data indicate that ProDTC at concentrations ranging from 100 to 500 μM inhibits NF-κB binding activity induced by oxidative stress in the form of PV.

Figure 22 illustrates the comparative effects of PDTC and Pro-DTC on regional myocardial ischemia in an ischemia-reperfusion model. These data show that ProDTC administration results in a significantly greater reduction in myocardial infarct size in comparison to PDTC.

Description of the Specific Embodiments

The invention described herein provides methods for treating a diverse array of inflammatory responses, conditions, and disease states in a mammalian patient. The methods of the invention involve administering an antiinflammatory effective amount of a cyclic dithiocarbamate (DTC) or thiuramsulfide (TS) derivative.

Preferred methods within the invention involve administering an antiinflammatory effective amount of a cyclic DTC or TS derivative to specifically inhibit NF-κB activity and/or oxidative stress while not substantially inhibiting other pathways involved in inflammation. Among other benefits, the methods of the invention leave intact the patient's ability to fight infection.

Cyclic DTC or TS derivatives that are useful within the invention exhibit antiinflammatory activity. As used herein, antiinflammatory activity means that the compound detectably or substantially inhibits one or more targeted inflammatory responses. These responses are measured by standard, *in vitro* or *in vivo*, assays, eg., cellular toxicity assays, pathology assays, immunohistochemical assays, cytokine assays, physiological assays (eg., blood pH, arterial pressure, and heart rate), and the like, which assays are generally known in the art.

Useful assays for determining antiinflammatory activity, for determining an "antiinflammatory effective amount" of compounds for use within the methods of the invention, and for evaluating the efficacy of methods disclosed herein include, but are not limited to, all of the assays incorporated in the examples below, as well as all assays set forth in the references cited herein (all of which are

incorporated herein by reference in their entirety for all purposes to the same extent as if each of said references were incorporated individually in local and specific context) or otherwise known in the art.

Inflammatory responses which can be measured for determining antiinflammatory activity, for determining an antiinflammatory effective amounts of compounds, and for evaluating the efficacy of methods of the invention include, but are not limited to, all measurable parameters indicative of the level of risk or the extent of inflammatory activity or related injury associated with: ischemia-reperfusion injury, complications of cardiopulmonary bypass or extracorporeal membrane oxygenation (ECMO), systemic inflammatory induced shock syndrome (eg., associated with CPB, ECMO, shock, sepsis, or pancreatitis), intimal hyperplasia, atherosclerosis, arthritis, chronic inflammatory autoimmune disease, inflammatory skin disease, periodontal disease, inflammatory pulmonary injury (eg., associated with ARDS, Asthma, COPD), gastrointestinal inflammatory disorders, and adverse sequelae associated with surgical procedures or injury, among other inflammatory conditions and disease states.

Antiinflammatory effective compounds, and antiinflammatory effective amounts of compounds for determining dosages within the methods of the invention are capable of yielding, or yield, an antiinflammatory response as defined above which is detectable compared to an accepted control value or response. For example, in an ischemia/reperfusion assay, an IL-1-induced inflammation or shock assay, or a cytokine assay (as described in the examples below), the compound (or antiinflammatory effective amount thereof) will yield a detectable antiinflammatory response as compared to a relevant control value (eg., the value of a parameter measured in an untreated sample, or a baseline physiological measurement).

In preferred aspects of the invention, compounds that exhibit antiinflammatory activity (or antiinflammatory effective amount thereof) are defined as yielding a "substantial" antiinflammatory response. A substantial

response, will vary at least 10% (above or below depending upon the parameter being measured) from the relevant control value. More preferably, the antiinflammatory response will vary 10-20%, sometimes 20-50%, and most preferably 50-100%, 100-200%, or more, compared to the relevant control value.

Antiinflammatory effective compounds within the invention comprise a discrete group of structurally and functionally related DTC and TS derivatives. These derivatives are preferably synthesized using a cyclic, secondary amine. Cyclic DTCs for use within the invention preferably share the general formula R1,R2-N-C(S)S-R3, wherein R1 and R2 constitute a cycle including the nitrogen atom of the dithiocarbamate. Preferred TS derivatives share the general formula R1,R2-N-C(S)-Sx-C(S)-N-R3,R4, wherein x is 1 to 8, and wherein R1, R2 and R3, R4 constitute independently a cycle including the nitrogen atoms of the respective dithiocarbamate residue.

Operative DTC and TS derivatives within the invention may have 5- to 7-membered ring structure(s), with 4- to 6-membered bridge component(s). The ring may contain either zero, one, or two double bonds. Preferred members of each group bear one or more, and preferably one or two substitutents in the bridge portion(s), eg., R4,R5 or R5, R6. The substituents may be selected from carboxyl, carboxylester, hydroxyl, nitro, amino or mercapto substitutents.

Other preferred DTC and TS derivatives for use within the invention are characterized by having, alternatively or in addition to the above noted substituent(s), one or more heteroatoms (S, N, or O) within the bridge portion(s). Additional preferred derivatives may feature, alternatively or in combination with the above noted substituent(s) and/or heteroatom(s), one or two carbonyl functions. Still other preferred derivatives feature, alternatively or in combination with the above noted substituent(s) and/or heteroatom(s) and/or carbonyl function(s), dehydrogenation to yield one or two double bonds.

Characteristic of preferred DTC and TS derivatives for use within the invention is that they exhibit

pharmacological properties unique to cyclic compounds and are more hydrophilic as compared to hydrophilicity of a parent compound lacking the above noted substituent(s) and/or heteroatom(s) and/or carbonyl function(s) and/or dehydrogenation features.

Exemplary DTC and TS derivatives exhibiting the aforementioned structural and functional characteristics to yield therapeutic agents for use within the methods of the invention include prolinedithiocarbamate (ProDTC), thioproline-dithiocarbamate (TProDTC), prolinethiuramidisulfide (ProTDS), and thioprolinethiuramidisulfide (TProTDS).

The precise amounts, frequency, and duration of treatment will depend on the status of the inflammatory condition or disease and on other factors such as the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. These factors will vary such that specific regimens can be established by those skilled in the art to maximize efficacy of treatment. Ordinarily, the cyclic DTC or TS derivative is administered in a dosage of between about 0.5 and 500 mg/kg body weight. Preferably, the dosage is between about 10 mg/kg and 100 mg/kg, and more preferably between about 20 mg/kg and 50 mg/kg. The administration schedule can range from a continuous infusion, to once every other day, to 6 or more administrations a day, with dose levels and administration protocols being selected by the health professional.

Thus, treatments according to the invention can be in the form of a one time dose, as in the case of a trauma patient in shock or heart surgery patient. Alternatively, short term, long term, or continuous treatments may be selected. For example, long term treatments can be employed to prevent chronic oxidative stress induced injury, to treat advanced lesions, or to manage high-risk patients. Long term treatments can extend for years with dosages ranging from 0.5 to 500 mg/kg body weight administered at intervals ranging from once every other day to three times daily.

The active compounds can also be administered in a period that is concurrent with or closely preceding a surgical procedure or other event anticipated to produce a risk of inflammatory injury, for example prior to coronary bypass. Thus, methods are provided which involve administration of a cyclic DTC or TS derivative concurrent with, or within an anti-inflammatory effective period preceding, a surgical procedure, whereby the administration reduces or eliminates risk of abnormal inflammatory responses normally associated with the procedure.

Within the methods of the invention, a cyclic DTC or TS derivative, or a mixture of these and/or other compounds, can be administered by a variety of routes, including intravenously, orally, intrathecally, topically to the eyes, skin or mucous membranes, in the form of ear or nasal drops, rectally or as part of a *ex-vivo* organ preservation solution. The cyclic DTC or TS derivative or mixture can also be administered directly to a blood vessel wall or within a lumen of a vessel, eg., during a catheter directed interventional procedure.

For example, an active compound or mixture as set forth herein can be administered directly to a vascular wall using perfusion balloon catheters, following or in lieu of coronary or other arterial angioplasty. In one such exemplary method, 2-5 mL of a physiologically acceptable solution containing about 1 to 500 μM of the compound or mixture is administered at 1-5 atmospheres pressure to treat or prevent restenosis following angioplasty, followed by long-term administration using alternative routes as described above.

Methods of the invention alternatively involve administration of a prescribing a dietary supplement containing a cyclic DTC or TS derivative, eg., in the form of total parenteral nutrition (TPN) and enteral feeds. In this aspect of the invention, DTC or TS derivatives can be administered in conjunction with other nutrients, such as Vitamin E, Co-enzyme Q10, Vitamin C, Vitamin A, Bioflavonoids, Ginseng, Grape skin extract, rutin, Zinc, Thiamin, biboflavin, niacinamide,

pantothenic acid, pyridoxine, folic acid, biotin, cynacobalamin and other antioxidants.

Other methods within the invention involve administration of a cyclic DTC or TS derivative in conjunction with other medications used in the treatment of inflammatory disease and related conditions, such as cardiovascular disease. Other medications useful in these combinatorial treatment methods include steroidal antiinflammatories, such as corticosteroids, and nonsteroidal antiinflammatories, for example aspirin, ibuprofen, indomethacin, fenoprofen, mefenamic acid, flufenamic acid, and sulindac. Additional adjunctive medicaments can include clotting inhibitors such as heparin; antithrombotic agents such as coumadin; calcium channel blockers such as varapamil, diltiazem, and nifedipine; angiotensin converting enzyme (ACE) inhibitors such as captopril and enalopril, and β-blockers such as propanalol, terbutalol, and labetalol, among other combinatorially effective medicaments which will be apparent to the skilled practitioner.

Typically, the cyclic DTC or TS derivative will be administered in the form of a pharmaceutical composition dissolved or suspended in a physiologically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. Many other suitable carriers are known in the art and readily formulated with the subject compounds, including biologically compatible gels and the like suitable for topical administration. The pharmaceutical compositions may be sterilized by conventional, well known sterilization techniques. The resulting formulations may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

Oral compositions for use within the invention will generally include an inert diluent or an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active

compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition.

The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents.

The cyclic DTC or TS derivative (or pharmaceutically acceptable salt, complex, or derivative thereof) can be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediarninetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parental preparation can be enclosed in ampoules,

disposable syringes or multiple dose vials made of glass or plastic.

The active compound can also be administered through a dressing or transdermal patch. Methods for preparing transdermal patches are known to those skilled in the art. For example, see Brown, L., and Langer, R., Transdermal Delivery of Drugs, Annual Review of Medicine, 39:221-229 (1988), incorporated herein by reference.

In other embodiments of the invention, the cyclic DTC or TS derivative is prepared with carriers that protect the compound against rapid elimination from the body, such as are routinely used in controlled release devices and formulations (eg., implants and microencapsulated delivery systems). Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

Liposomal suspensions may also be pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811 (incorporated herein by reference). For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound is then introduced into the container. The container is swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

The pharmaceutical compositions for use within the invention may also contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH-adjusting and buffering agents, tonicity-adjusting agents and the like, for example, sodium

acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. The concentration of the cyclic DTC or TS derivative in these formulations can vary widely, i.e., from less than about 0.5%, usually at least about 1%, to as much as 15 or 20% by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

In preferred methods within the invention, humans, and other animals, in particular, mammals, suffering from inflammatory conditions and diseases are treated by administering to the patient a pharmaceutical or therapeutic composition comprising an antiinflammatory effective amount of one or more cyclic DTC or TS derivatives, or a pharmaceutically acceptable salt, derivative, or complex thereof, in a pharmaceutically acceptable carrier or diluent.

As used herein, the term "pharmaceutically acceptable salts or complexes" refers to salts or complexes that retain the desired biological activity of the cyclic, substituted DTC or TS derivative and exhibit minimal undesired toxicological effects. Nonlimiting examples of such salts are (a) acid addition salts formed with inorganic acids (for example, hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like), and salts formed with organic acids such as acetic acid, oxalic acid, tartaric acid, succinic acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, naphthalenedisulfonic acid, and polygalacturonic acid; (b) base addition salts formed with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, sodium, potassium, and the like, or with an organic cation formed from N,N-dibenzylethylene-diamine, Dglucosamine, ammonium, tetraethylammonium, or ethylenediamine; or (c) combinations of (a) and (b); e.g., a zinc tannate salt or the like.

The active compound is included in the pharmaceutically acceptable carrier or diluent in an amount sufficient to

deliver to a patient an antiinflammatory effective amount without causing serious toxic effects in the patient treated. The active compound is preferably administered to achieve peak plasma concentrations of the compound of about 0.1 to 100 μM, preferably about 1-10μM.

The concentration of active compound in the drug composition will depend on absorption, distribution, inactivation, and excretion rates of the drug, as well as other factors known to those of skill in the art. It is to be noted that dosage values will also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

In view of the description and examples provided herein, the methods of the invention are shown to be useful for treating a wide variety of inflammatory conditions and diseases. In particular, the methods of the invention can be employed to treat ischemia-reperfusion injury, and for preventing or reducing related complications of cardiopulmonary bypass and extracorporeal membrane oxygenation (ECMO). In addition, the methods provided herein are useful to prevent or alleviate systemic inflammatory induced shock or response syndrome associated with CPB, ECMO, shock, sepsis, severe pancreatitis and other injuries or disease states.

Additional methods within the invention incorporate the foregoing treatments to prevent or reduce inflammatory conditions or diseases, including but not limited to, intimal hyperplasia; atherosclerosis; arthritis (osteo and rheumatoid); chronic inflammatory autoimmune diseases (eg., multiple sclerosis); inflammatory skin disease; periodontal disease; pulmonary injury (eg., ARDS, Asthma, COPD); GI inflammatory

disorders (eg., inflammatory bowel disease, volvulus, ischemic gut, Crohn's disease);

Related methods within the invention incorporate the foregoing treatments to prevent or reduce adverse sequelae associated with surgical procedures or other injury, including but not limited to ischemic injury and/or rejection from transplantation (eg., free tissue transfer and reimplantation of severed digits and extremities), ischemic injury from testicular or ovarian torsion, hypoxic cerebrovascular injury, reocclusion after thrombolysis for acute myocardial infarction or thrombosis after microvascular reconstruction, stroke, vasospasm (eg., after aneurysm clipping, prinzmetals variant angina, CABG, or free tissue transfer for reconstructive surgery).

Yet additional methods within the invention incorporate the foregoing treatments to prevent or reduce conditions that are exacerbated, either primarily or secondarily, by inflammatory conditions, including infertility, the occurrence and size of heart attacks, cancer (malignant angiogenesis), complications from diabetes (eg., eye, kidney, neuropathy), complications of chemotherapy, and complications of AIDS.

The methods of the invention effectively inhibit (i.e., prevent or treat) oxidative stress-induced tissue injury *in vivo*, including injuries in the heart, brain, liver, kidneys, bone, immune system, skin, and other organs. In addition, the methods of the invention are useful to alleviate arrhythmia, lung injury, and other adverse sequelae caused by oxidative stress. These methods involve administration of an anti-oxidative stress effective amount of a cyclic DTC or TS derivative. Inhibition of oxidative stress is determined according to any of a variety of models and methods known in the art, for example based on well established *in vitro* or *in vivo* protocols measuring cellular indices, physiological conditions, and other parameters indicative of the risk, or extent, of oxidative stress-related injury.

The methods of the invention preferably achieve substantial inhibition of oxidative stress responses or injuries attributable thereto. Thus, administration of an anti-oxidative stress effective amount of a cyclic DTC or TS derivative in a clinical, ischemia/reperfusion (I/R) setting preferably results in at least a 10% reduction, more preferably a 20-30% and in some cases a 50-80% reduction of I/R related physiological responses or tissue injury compared to the relevant control value.

Other preferred methods within the invention involve administration of a cyclic DTC or TS derivative, for example, ProDTC or PRoTDS, in an amount effective to prevent or treat systemic inflammatory response syndrome (SIRS). These and related methods involve administering, by local, regional, systemic or *ex-vivo* perfusion, to a tissue of the patient at risk or subject to inflammatory injury (eg., tissue subject to interval tissue ischemia) an anti-SIRS effective amount of the cyclic DTC or TS derivative to a mammalian patient. This treatment yields a measurable reduction, preferably a substantial reduction, in the risk or extent of the SIRS response.

In still other preferred aspects of the invention, methods are provided which employ a cyclic DTC or TS derivative that is contacted with cells or administered to a patient to specifically inhibit NF-κB activity. These methods result in detectable or substantial inhibition of one or more biological activities of NF-κB, including but not limited to, NF-κB nuclear translocation, release of an inhibitory subunit, IκB, of NF-κB, NF-κB-mediated transcriptional activation, and/or NF-kB-mediated inflammatory responses.

For example, in an accepted *in vitro* or *in vivo* model for NF-κB activation (eg., an LPS activation model), an anti-NF-κB effective amount of a cyclic DTC or TS derivative is administered to yield detectable inhibition of NF-κB nuclear translocation (see Examples, below). Alternative methods yield detectable inhibition of the release of IκB, or of NFκB-mediated transcriptional activation (see, eg., Schreck et al.,

J. Exp. Med. 175:1181-1194, 1992, incorporated herein by reference). In related methods, NF-κB-mediated transcriptional regulation of selected genes, eg., tissue factor (TF), involved in specific inflammatory pathways or mechanisms is detectably or substantially inhibited.

In more detailed methods within the invention, inhibition of an NF-κB activity is achieved without substantial degradation of IκBα (see Examples below). By "substantial" degradation is meant that IκBα is not degraded by more than 50%, preferably by no more than 20-30%, and more preferably by no more than 5-10%, over control values in samples exposed to an anti-NF-κB effective amount of a DTC or TS derivative compared to controls. Thus, in related methods a cyclic DTC or TS derivative is administered to differentially inhibit NF-κB-mediated oxidative stress without affecting other inflammatory mechanisms and/or pathways that would detectably or substantially reduce desired responses, such as one or more of the beneficial responses elicited by TNF or LPS, thereby leaving the patient's ability to fight infection.

In other aspects of the invention, specific methods are provided for inhibiting inflammatory responses without substantially elevating adverse inflammatory-stimulating chemokines (eg., IL-8, MCP-1, and GRO). Within these methods, a cyclic DTC or TS derivative is contacted with a cell or administered to a patient in an antiinflammatory effective amount which does not detectably or substantially yield an increase in an inflammatory-stimulating chemokine. In this context, preferred methods yield a maximal increase in inflammatory-stimulating chemokine levels (eg., as measured in plasma following IL-1α or LPS stimulation) of less than 50% following administration of the cyclic DTC or TS derivative. Preferably, the maximal increase is no more than 20-30%. More preferably, an increase of less than 10% follows administration of the antiinflammatory compound. Even more preferably, administration of the compound attenuates levels of inflammatory-stimulating chemokines in the context of an ongoing inflammatory response (eg., in a patient experiencing

sepsis or in a control sample stimulated by IL-1 or LPS) by 30-50% or more.

In accordance with the foregoing methods, *in vivo* administration of the cyclic DTC and TS derivatives of the invention preferably yield a maximum level of IL-8 of 0.3 ng/ml, more preferably 0.25 ng/ml, and most preferably 0.1 ng/ml or lower. Preferred levels of GRO elicited or attenuated by the above methods are no greater than 4 ng/ml, preferably no greater than 3 ng/ml, and most preferably 1 ng/ml or less. For MCP-1, preferred levels are less than 12 ng/ml, more preferably 8 ng/ml or less, and most preferably 5 ng/ml or less.

Yet additional methods are provided herein which involve administration of a cyclic DTC or TS derivative, alone or in a mixture, to preserve organs and tissues *ex-vivo* for purposes of transplantation or chemotherapy. Within this aspect of the invention, the organ or tissue is contacted with an effective amount of the cyclic DTC or TS derivative, eg., in a perfusate, to reduce or prevent inflammatory injury normally associated with *ex vivo* tissue or organ maintenance. For example, an organ being prepared for transportation and transplantation can be perfused, once, repeatedly, or continuously, with a preservation solution under normal or hypothermic conditions, to measurably reduce tissue damage expected under control (i.e., perfused without the effective compound or mixture) conditions.

The cyclic DTC or TS derivatives and pharmaceutical formulations administered within the methods of the invention must be physiologically acceptable. In general, compounds and formulations with a therapeutic index of at least 2, preferably at least 5-10, more preferably greater than 10, are acceptable. As used herein, the therapeutic index is defined as the EC_{50}/IC_{50}, wherein EC_{50} is the concentration of compound that provides 50% inhibition of a target inflammatory response (eg., lymphokine-induced inflammation and hypotension, lymphokine-induced nuclear translocation of NF-κB; inflammation-associated increases in chemokine levels; ischemia-reperfusion injury; or systemic inflammatory-induced shock) compared to a relevant

control, and IC_{50} is the concentration of compound that is toxic to 50% of target cells, eg., in an *in vitro* toxicity assay. In this context, cellular toxicity can be measured by direct cell counts, trypan blue exclusion, or various metabolic activity studies such as ^{3}H-thymidine incorporation, as known to those skilled in the art.

In more detailed aspects of the invention, the methods set forth above reduce or prevent an acidotic response in a patient or sample so as to alleviate a principal toxic side effect associated with administration of DTC compounds other than the cyclic derivatives disclosed herein. In this context, preferred methods yield a maximal decrease in pH in *in vivo* applications to yield an arterial pH of about 7.1, preferably about 7.2, and more preferably about 7.3-7.45 or higher.

A substantial response, will vary at least 10% (above or below depending upon the parameter being measured) from the relevant control value. More preferably, the antiinflammatory response will vary 10-20%, sometimes 20-50%, and most preferably 50-100%, 100-200%, or more, compared to the relevant control value

The methods of the invention provide substantial, unexpected advantages over prior methods which have used different DTCs, for example non-cyclic DTCs as exemplified by pyrrolidine-DTC (PDTC), as potential agents to treat various conditions, including inflammation. In contrast to prior methods, eg., methods employing PDTC and other agents, preferred methods of treatment within the invention involve administration of a cyclic DTC or TS derivative, for example, ProDTC or PRoTDS, in an amount effective to selectively block specific inflammatory pathways or mechanisms, including specific NF-κB activities (eg., translocation without IκB degradation) and to selectively inhibit oxidative stress, while not significantly inhibiting other pathways or mechanisms, such as other pathways and mechanisms mediated by TNF and/or LPS-induced activation, and without eliciting other adverse

sequelae, such as are attributable to deleterious cytokine induction.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE I

Structure and Synthesis of Active Compounds

Active compounds that are useful within the invention have been identified to include a discrete family of structurally and functionally related, cyclic derivatives of dithiocarbamates (DTCs) and thiuramsulfides (TSs). These related compounds actively inhibit a range of inflammatory responses, particularly responses mediated by NF-κB activity and/or oxidative stress.

Operable DTC and TS derivatives within the invention are preferably synthesized using a cyclic, secondary amine, for example a cyclic, secondary amino acid such as proline or thioproline. Non-limiting examples of cyclic, secondary amine precursors for synthesis of cyclic derivatives of DTCs and TSs for use within the invention are provided in Figures 1A and 1B. For synthesis of preferred compounds within the invention, the precursors shown in Figure 1A are substituted, as discussed below, whereas the exemplary precursors shown in Figure 1B need not be substituted to yield a preferred DTC or TS derivative.

Cyclic DTCs useful within the invention preferably share the general formula R1,R2-N-C(S)S-R3, wherein R1 and R2 constitute a cycle including the nitrogen atom of the dithiocarbamate. The ring may be 5-, 6-, or 7-membered, with a 4- to 6-membered bridge, and may contain either zero, one, or two double bonds. Members of this group of compounds preferably bear one or more, and preferably one or two substitutents in the bridge portion, eg., R4 and R5. Thus, the bridge may have a structure $(CH_2)^n$ (n = 4-6), wherein the bridge is substituted at least at one $-CH_2-$ group. The substituents may be selected from a variety of known substituents, eg., carboxyl, carboxylester, hydroxyl, nitro, amino or mercapto

substitutents. Non-limiting examples of preferred substituents are shown in Figure 2. These and other substitutents may be combined (eg., by substitution at a CH_2 or N within the bridge) with a cyclic, secondary amine (eg., with any of the cyclic, secondary amines shown in Figures 1A and 1B) to yield a preferred, cyclic, substituted secondary amine precursor for synthesis of DTC and TS derivatives useful within the invention. Non-limiting examples of cyclic, substituted secondary amines for synthesis of preferred DTC and TS derivatives within the invention are provided in Figures 3 and 4.

Other members of the group of DTC derivatives that are useful within the invention feature, alternatively or in addition to the above noted substituent(s), one or more heteroatoms (S, N, or O) within the bridge portion. Yet additional members feature, alternatively or in combination with the above noted substituent(s) and/or heteroatom(s), one or two carbonyl functions. Still other members feature, alternatively or in combination with the above noted substituent(s) and/or heteroatom(s) and/or carbonyl function(s), dehydrogenation to yield one or two double bonds. In more detailed aspects, R3 is a sodium, potassium, or free or substituted ammonium.

Characteristic of preferred DTC derivatives for use within the invention is that they are more hydrophilic as compared to hydrophilicity of a parent compound lacking the above noted substituent(s) and/or heteroatom(s) and/or carbonyl function(s) and/or dehydrogenation features.

The second group of compounds useful within the invention include cyclic thiruamsulfides, which can incorporate one or more sulfurs, eg., thiuramsulfides (TDs), thiruramdisulfides (TDSs), or thiuramtrisulfides (TTSs). Preferred members of this group of compounds share the general formula R1,R2-N-C(S)-Sx-C(S)-N-R3,R4, wherein x is 1 to 8, and wherein R1, R2 and R3, R4 constitute independently a cycle including the nitrogen atoms of the respective dithiocarbamate residue. Accordingly, R1, R2 may, or may not, form an

identical bridge as R3, R4. In certain aspects of the invention, bifunctional heterocycles are preferred.

Also within this second group of compounds (TS derivatives), the ring(s) may be 5- to 7-membered, with a 4- to 6-membered bridge, and may contain either zero, one, or two double bonds. Preferred members of this group likewise bear one or more, and preferably one or two substitutents in the bridge portion(s), eg., R5 and R6. Thus, the bridge(s) may have a structure $(CH_2)^n$ (n = 4-6), wherein the bridge is substituted at least at one $-CH_2-$ group. The substituents may be selected from carboxyl, carboxylester, hydroxyl, nitro, amino or mercapto substitutents. Other members of this group feature, alternatively or in addition to the above noted substituent(s), one or more heteroatoms (S, N, or O) within the bridge portion. Yet additional members feature, alternatively or in combination with the above noted substituent(s) and/or heteroatom(s), one or two carbonyl functions. Still other members feature, alternatively or in combination with the above noted substituent(s) and/or heteroatom(s) and/or carbonyl function(s), dehydrogenation to yield one or two double bonds.

As in the case of the DTC derivatives discussed above, non-limiting examples of cyclic, secondary amine precursors for synthesis of cyclic derivatives of TSs for use within the invention are provided in Figures 1A and 1B. Non-limiting examples of preferred substituents are shown in Figure 2. Non-limiting examples of cyclic, substituted secondary amines for synthesis of preferred TS derivatives for use within the invention are provided in Figures 3 and 4.

Also characteristic of preferred TS derivatives for use within the invention is that they are more hydrophilic as compared to hydrophilicity of a parent compound lacking the above noted substituent(s) and/or heteroatom(s) and/or carbonyl function(s) and/or dehydrogenation features.

Synthesis of cyclic DTC derivatives for use within the invention preferably involves reaction of a cyclic, secondary amine, preferably a cyclic, substituted, secondary amine, with carbon disulfide (CS_2) in alkaline, ethanolic

solution (see, eg., Frank et al., Carcinogenesis 11:199-203, 1990, incorporated herein by reference). Other modifications, eg., to provide alternate or additional features including substituent(s), heteroatom(s), carbonyl function(s), and/or dehydrogenation, are provided in accordance with conventional methods and will depend on starting materials (eg., structure of the secondary amine precursor), desired end products, and other factors understood by the artisan. In this context, it is further understood that the synthetic reaction can utilize a variety of bases and salts. For example, using ammonia as a base, the reaction may yield an ammonium salt of a dithiocarbamic acid. Figure 5 depicts an exemplary synthetic reaction (and putative reaction mechanism) of CS_2 and L-proline in ethanol and NH_3 to yield a salt of a preferred DTC derivative, prolinedithiocarbamate (ProDTC) for use within the invention.

As described in detail above, a range of useful DTC derivatives are contemplated within the invention that are synthesized using alternate, cyclic, secondary amines. Figure 6 illustrates other, non-limiting examples of reactions yielding preferred DTC derivatives for use within the methods of the invention. Specifically, the Figure illustrates an exemplary reaction of piperidine-4-carbonic acid with CS2 to yield N,N-(3-carboxy-pentamethylene) dithiocarbamate, and exemplary synthesis of a multiply substituted DTC derivative, N,N-(1-carboxy-3-hydroxy)-tetramethylene-dithiocarbamate, from 4-hydroxy-trans-proline.

Preparation of cyclic, substituted TS derivatives for use within the invention involves oxidation of a corresponding DTC compound using an appropriate oxidan (see, eg., Kitson, Biochem J. 155:445-448, 1976, incorporated herein by reference). For example, Pro-TDC-sodium salt may be oxidized with iodine/potassium iodide to yield a preferred TS derivative, prolinethiuramdisulfide (Pro-TDS), for use within the invention. A more preferred oxidan is nitrogendioxid (N_2O_4) which renders excess reactants volatile and saves a clean up step.

As in the case of synthesis of DTC derivatives, synthesis of TS derivatives for use within the invention can utilize a variety of conventional modifications, eg., to provide alternate or additional features including substituent(s), heteroatom(s), carbonyl function(s), and/or dehydrogenation, depending on starting materials, desired end products, and other factors. Likewise, alternative bases, salts, and reaction conditions can be used in accordance with ordinary chemical practice.

EXAMPLE II

Dithiocarbamates Attenuate Endotoxin and IL-1-Induced Hypotension *In Vivo*

Despite advances in critical care and in the surgical treatment of severe infection, cytokine-mediated shock remains a significant contributor to morbidity and mortality among hospitalized patients. Lynn et al., Clin. Infect. Dis. 20:143-158, 1994, incorporated herein by reference. Although the most familiar form of this syndrome is the association between gram-negative bacteria and septic shock, a similar syndrome can be found in a variety of clinical settings including resuscitated patients following shock, pancreatitis, and the whole body inflammatory response to cardiopulmonary bypass. Cytokine-induced shock is characterized clinically by a reduction in mean arterial pressure (MAP), an increase in heart rate, an increase in cardiac output, and a marked reduction in systemic vascular resistance. The result is an inability to adequately perfuse end organ tissue and the development of multiple system organ failure and death.

A variety of studies indicate that interleukin 1 (IL-1) is a principle component of the systemic inflammatory response syndrome (SIRS). IL-1 synthesis occurs early in the inflammatory response and thus has a proximal role in the induction of other proinflammatory cytokines. IL-1 production has been directly linked to the development of hypotension, shock, multi-organ failure, hematologic dyscrasia, and death in experimental models and patients with SIRS.

The pathophysiology of cytokine-induced shock is thought to be mediated by inducible changes at the microvascular level that occur when the endothelium is exposed to circulating cytokines. Quiescent endothelial cells lining the microvasculature respond to inflammatory stimuli and cell injury by activating signal transduction pathways that transmit signals through the cytoplasm to the nucleus. These signals result in the transcription, translation, and expression of several genes involved in SIRS.

Deletional and mutational analysis of several proinflammatory, procoagulant, and vasoactive genes activated in this process (IL-1, E-selectin, vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), interleukin-8 (IL-8), monocyte chemotactic protein-1, inducible nitric oxide synthase (iNOS), and tissue factor) indicate that these genes are transcriptionally regulated in a process that requires nuclear binding of a single family of transcription factors, nuclear factor-κB (NF-κB). Baeuerle et al., Ann. Rev. Immunol. 12:141-79, 1994; Baeuerle et al., Cell 87(1):13-20, 1996; Baeuerle et al., Immunol. 65:111-37, 1997, each incorporated herein by reference.

NF-κB is a ubiquitous, inducible, fast-responding transcription factor that exists in an inactive form in the cell cytoplasm. In response to a diverse array of extracellular inflammatory signals *in vitro*, NF-κB is translocated to the nucleus where the now activated NF-κB binds to specific nucleotide sequences in the promoter region of these genes and, in association with other transcription factors, initiates gene expression (reviewed in Baeuerle et al., Ann. Rev. Immunol. 12:141-79, 1994, incorporated herein by reference). These DNA binding proteins are thought to facilitate the assembly of higher order complexes of transcriptional activators that interact as a unit with the basal transcriptional machinery needed to initiate transcription. Thanos et al., Cell 80:529-532, 1995, incorporated herein by reference.

NF-κB is activated *in vivo* at sites of inflammation in response to bacterial lipopolysaccharide (LPS), and this activation temporally precedes the expression of E-selectin, ICAM, and VCAM at these sites. This suggests a role for NF-κB in the activation of these genes *in vivo* during severe systemic inflammation. It is unknown, however, if inhibiting NF-κB will prevent the shock-like state that is associated with SIRS. In this example, the ability of dithiocarbamates, and cyclic, dithiocarbamate derivatives, represented by the exemplary group members PDTC, and ProDTC, that reportedly or demonstrably inhibit NF-κB, to attenuate IL-1-induced shock *in vivo* is demonstrated.

A) Rabbit model

New Zealand White rabbits weighing 3 to 4 kg were used in research protocols approved by the Animal Care Committee of the University of Washington, Seattle. All animals received humane care according to the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 85-23). The rabbits were anesthetized with an initial intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg). They were intubated using an endotracheal tube (inner diameter 3.0 mm) and maintained on inhaled Halothane anesthesia (1%) in 1010% oxygen with a tidal volume of 15 cc/kg for the duration of the study using a small animal ventilator. Minute ventilation was adjusted according to arterial blood gas measurements to maintain arterial pH between 7.45 and 7.50 while keeping carbon dioxide tension between 25 and 35 mm Hg. Body temperature was maintained at 36° to 38° C with a heating pad. Following a standard cut-down technique, a 20-gauge flexible catheter was placed in the left common carotid artery to continuously measure heart rate (HR), mean arterial pressure (MAP), and collect blood samples. A double lumen thermodilution probe and injectate catheter (Model 94-011-3F, Baxter Healthcare Corporation, Irvine, CA) were placed through the left femoral artery and vein in order to measure core temperature and cardiac output (CO) by

thermodilution. A maintenance drip of lactated Ringer's solution (4-5 ml/kg/h) was administered and central venous pressure measured via a 20-gauge flexible catheter inserted into the right external jugular vein. An additional 30 to 50 cc of normal saline was administered through the flushing of lines to maintain patency throughout the experiments. The cardiac output determinations were performed in triplicate and the results averaged. Systemic vascular resistance (SVR) was calculated as follows: (MAP-CVP)80/CO (dyn ∘ s ∘ cm^{-5}). After insertion of the catheters, rabbits were allowed to stabilize for 30 to 60 minutes before baseline values were obtained. Minute ventilation and tidal volume were not changed after this baseline was established.

B) Study groups

24 rabbits were randomly assigned to four groups (n=6 per group) and monitored for 240 minutes. Animals were treated with 1.0 mL phosphate buffered saline (PBS) and either PDTC or ProDTC (15 mg/kg in 1 mL of PBS) i.v. 60 minutes prior to i.v. infusion of IL-1a (5mg/kg; provided by Frank Chizzonite, Ph.D., Hoffmann-LaRoche Inc., Nutley, NJ.) or 1.0 mL saline alone. Group 1 received PBS followed by saline. Group 2 was given PBS prior to IL-1a infusion. Group 3 received PDTC before the administration of IL-1a. Group 4 was treated with ProDTC prior to IL-1a infusion. Blood samples (0.5-1.0 ml) were taken from the carotid catheter 60 minutes before IL-1α or saline administration, and at time 0, and 30, 60, 120, 180, and 240 minutes after saline or IL-1α administration, and the volume of blood taken was replaced by an equal volume of saline.

C) Statistical Analysis

All data analysis herein was performed using SPSS for Windows, version 6.1. For each of the treatment groups, several parameters (expressed as continuous variables) were studied at the indicated time points. To determine if a statistically significant change occurred compared with a baseline value, these parameters were compared with that value using paired t-tests. The baseline time point occurred at t =

0 for the hemodynamic and hematologic parameters, but occurred at t = -60 for the acid/base parameters, since the acid/base was found to be profoundly affected by PDTC. For intergroup comparisons, the mean change (from baseline) for each of these parameters was analyzed at the specified time points using ANOVA. Fisher's Least Significant Difference test was used for post-hoc comparisons. P-values less than 0.05 were considered statistically significant.

RESULTS

Animals in the control group treated with saline only remained hemodynamically stable throughout the 360-minute observation period. Those rabbits receiving only PBS prior to LPS became progressively hypotensive and tachycardial during the course of the experiments in response to the LPS infusion. Pretreatment with PDTC one hour prior to LPS infusion significantly attenuated the LPS-induced hypotension that developed in control animals after 240 minutes (Figure 7; $P < 0.005$). This effect remained significant for the duration of the study period ($P < 0.05$). PDTC had little effect on the increase in heart rate stimulated by LPS (Figure 8). One animal in the PBS/LPS control group died 300 minutes after LPS infusion. All of the animals in the saline control and PDTC treated groups survived the duration of the 6-hour observation period (survival was not a study endpoint).

Dithiocarbamate-mediated hemodynamic changes during systemic inflammation were further characterized in the IL-1 model. The infusion of IL-1 into Group 2 positive control animals resulted in a dramatic decrease in MAP that was maximal at 30 minutes (-21.6%±3.97%) and remained decreased throughout the 4 hour study period (Figure 9). This decrease in MAP was statistically significant from t = 30 to t = 120 minutes after the IL-1 infusion.

The MAP remained decreased by approximately 19% from 120-240 minutes but this drop was no longer significant compared to time 0 pressures after 120 minutes. When compared to rabbits of Group 1 that did not receive IL-1, the MAP

observed in Group 2 after IL-1 injection was significantly lower at every time point throughout the entire study period ($P < 0.05$). The change in the MAP of rabbits in Group 1 was not significant from time 0 at any time point.

The IL-1-induced fall in MAP was associated with a profound decrease in SVR as shown in Figure 10. The fall in SVR was significant at t = 30, 60, 120, 180, and 240 minutes after IL-1 infusion in Group 2. Those rabbits in Group 2 experienced a rapid increase in CO and HR after the IL-1 injection (Figures 11 and 12). The increase in CO in Group 2 animals reached significance and maximal change (34.4%±11.4%) at t = 120 minutes. CO in this group decreased toward baseline after 120 minutes but remained elevated for the duration of the study. HR in Group 2 increased steadily reaching significance at 60 minutes and remaining significantly elevated at the completion of the study period. Rabbits in Group 1 remained hemodynamically stable throughout the duration of the study.

When PDTC was administered 60 minutes before IL-1 infusion, the hypotensive response was reduced and delayed reaching a nadir at 60 minutes post-IL-1 injection. After 60 minutes the MAP approached baseline values but remained slightly depressed. The decrease in MAP was significant compared to t = 0 only at 30 and 60 minutes after IL-1 administration, and was not significant at any time point when compared to MAP in the saline control group (Figure 9). Treatment with ProDTC profoundly attenuated the IL-1 induced hypotension.

There was no significant difference in MAP between Group 4 and Group 1 although there was a brief decrease in MAP in Group 4 (-11.3% ± 6.5%) at 60 minutes that was significant when compared to t = 0 values. MAP quickly returned toward initial time 0 values in this group and remained elevated above baseline values at the end of the 4 hour study period (Figure 9). The MAP in Group 4 was significantly greater than that measured in Group 2 at 30 minutes ($P < 0.05$).

As might be expected based on the MAP data, the SVR in the PDTC group dropped significantly compared to t = 0 out

to 60 minutes showing an increase toward baseline with no significant difference from t = 0 or the saline control group observed after this time point. The SVR in the ProDTC group was significantly depressed compared to t = 0 at 60, 120, and 180 minutes, but remained elevated above the SVR calculated in Group 2 for the duration of the experimental protocol.

Rabbits in Group 2 experienced a drop in SVR that was significantly below the SVR measured in Group 3 and Group 4 at 30 and 60 minutes after IL-1 infusion ($P < 0.05$). The SVR of Group 2 continued to be significantly lower than that of the saline control animals in Group 1 for the duration of the experimental time period ($P < 0.05$). The SVR calculated in Group 3 was significantly elevated above that calculated in Group 2 at 120 and 180 minutes ($P < 0.05$).

Animals in Group 3 and Group 4 exhibited a transient, significant increase in CO during the first 60 and 120 minutes respectively following IL-1 treatment with return to baseline values after 120 minutes (Figure 11). CO measured in these groups was not significantly different from that of the saline controls in Group 1 at any time point. Rabbits in Group 2 experienced a dramatic rise in CO by 120 minutes that was significantly elevated above that measured in Group 1 after 120 minutes ($P < 0.05$).

HR significantly increased in Group 2 60 minutes after IL-1 infusion and remained significantly elevated in this group for the remainder of the study. The HR measured in Group 2 was significantly higher than that observed in Group 1 at 30 and 60 minutes ($P < 0.05$). Animals treated with PDTC in Group 3 experienced an immediate, profound increase in HR that was significantly elevated compared to t = 0 for the entire experiment. The tachycardia observed in Group 3 was significantly different from the HR of Group 1 and Group 4 at 60 and 120 minutes reaching a maximum increase (72.8% ± 2.3%) at 180 minutes where it was significantly greater than the HR observed in all of the other groups ($P < 0.05$). ProDTC significantly attenuated this reflex tachycardia for the first 180 minutes.

HR remained relatively unchanged in Group 4 and Group 1 with a similar slight increase at 240 minutes which was significant compared to time 0 for both groups (Figure 12). The HR in Group 4 never significantly differed from that measured in the saline control rabbits of Group 1. The HR in Group 4 was significantly slower than that measured in Group 2 only at 60 minutes ($P < 0.05$).

EXAMPLE III

PDTC But Not ProDTC Induces Unacceptable Levels of Metabolic Acidosis

The present example compares metabolic acidosis effects caused by *in vivo* administration of a dithocarbamate and a cyclic, dithocarbamate derivative, represented by the exemplary group members PDTC and ProDTC, respectively to alleviate interleukin 1 (IL-1)-induced hemodynamic instability.

Methods

In this example, rabbits were housed, anesthetized, maintained, and catheterized to measure core temperature and cardiac output (CO) as described above. Blood samples were removed and hemodynamic parameters and arterial blood gases were also recorded as above.

RESULTS

As demonstrated by the data presented in Figures, 13, 14, and 15, there were no significant differences in arterial pH, bicarbonate level, or calculated base deficit between the above noted groups before the administration of PDTC or ProDTC. However, the administration of PDTC generated a rapid and profound acidotic state. In particular, the rabbits receiving PDTC exhibited a significant, rapid fall in arterial pH and base deficit which was evident even before the infusion of IL-1 one hour later. Arterial pH and calculated base deficit were significantly lower in Group 3 compared to the other groups 60 minutes after PDTC treatment (Figures 13 and 14; $P < 0.05$).

In contrast with the PDTC study group, rabbits treated with either saline or ProDTC exhibited no significant change in pH, bicarbonate, or base deficit before IL-1 administration. Infusion of IL-1 precipitated a metabolic acidosis with a steady decrease in arterial pH, bicarbonate, and base deficit that was evident 60 minutes after receiving the IL-1. Animals in group 2 and group 4 had blood pH and base deficit levels that were significantly lower than those found in the saline control group 60 minutes after IL-1 administration (Figures 13 and 14; $P < 0.05$).

Arterial blood pH and base deficit levels measured in Group 2 and group 4 remained significantly below those of Group 1 after 180 minutes ($P < 0.05$). The calculated base deficit was not significantly different between these groups after 240 minutes. Measured arterial pH remained significantly lower in Group 2 compared to the pH measured in Group 1 saline controls after 240 minutes ($P < 0.05$). The arterial pH of those rabbits treated with ProDTC in Group 4 was no longer significantly lower than that of the saline control animals in Group 1 at the end of the experimental protocol (Figure 13). Rabbits in Group 3 continued to display a profound metabolic acidosis after IL-1 infusion with a sharp drop in pH and base deficit that reached a plateau after 30 minutes but remained well below the base deficit calculated in the other groups (Figure 14). The blood pH of animals receiving PDTC remained significantly below the level of that in the other groups out to 180 minutes after IL-1 injection ($P < 0.05$). At the end of the experimental time period differences in arterial pH and calculated base deficit level were no longer significant between Group 3 and Group 2. Arterial pH in Group 3 remained significantly lower than those values observed in Group 1 or Group 4 ($P < 0.05$). Calculated base deficit in Group 3 remained below that calculated in Group 4 after 240 minutes and continued to be significantly lower than that calculated in Group 1 ($P < 0.05$). Bicarbonate levels showed a downward trend in each group after IL-1 administration. The acid-base profile of the saline control group remained relatively stable over 5

hours with no significant decreases in pH, bicarbonate, or calculated base deficit observed (Figures 13, 14, and 15).

Summarizing the above results, PDTC and ProDTC both attenuated hemodynamic instability induced by IL-1 infusion. However, animals receiving PDTC treatment experienced a profound metabolic acidosis beyond that which was seen in untreated rabbits injected with IL-1 only, or treated with ProDTC. PDTC alone induced a significant acidosis that was evident even before the animals in this group were given IL-1. This acidotic state was manifest in the PDTC treated group within one hour of receiving the PDTC. ProDTC treatment was much less toxic.

The blood gas profile of the ProDTC treated animals was unchanged from that of saline control animals one hour after receiving the ProDTC. Animals treated with saline or ProDTC did not experience significant acidosis until after receiving IL-1, suggesting that the cytokine was primarily responsible for the moderate acidosis observed in this sample group.

ProDTC treatment did not immediately alter the cytokine-induced acidosis, as the acid-base status of the IL-1 control group and the ProDTC treated animals was almost identical up to 180 minutes after IL-1 infusion. Animals treated with ProDTC, however, experienced an increase in arterial pH after this point and no longer exhibited significant acidemia compared to saline control animals by the end of the experimental protocol. These changes cannot be attributed to differences in ventilation, as the ventilatory settings were not altered after similar and stable baseline blood gas measurements were attained in each group.

EXAMPLE IV

Chemokine Levels are Inhibited by ProDTC but not by PDTC

Inflammation is characterized by the accumulation of neutrophils and monocytes in tissue. Monocytic and endothelial cells orchestrate this response to injury in part by secreting chemotaxins that recruit neutrophils and monocytes into tissue.

IL-8, GRO, and MCP-1 are members of the chemokine family of proteins that are potent neutrophil and mononuclear cell chemoattractants and activators. Johnson et al., J. Biol. Chem. 271(18):10853-10858, 1996; Kajikawa et al., J. Immunol. Meth. 197:19-29, 1996, each incorporated herein by reference. In addition to their effects on these leukocytes, these chemokines have many other biological activities including regulatory effects on melanoma growth, fibroblast collagen production, activation of and adhesion to endothelial cells, myelopoiesis, and angiogenesis. Johnson et al., J. Biol. Chem. 271(18):10853-10858, 1996, incorporated herein by reference.

The present example compares the effects on chemokine levels caused by *in vivo* administration of a dithocarbamate and a cyclic, dithocarbamate derivative, represented by the exemplary group members PDTC and ProDTC, respectively.

Bioassay of chemokines in plasma

In this example, rabbits were housed, anesthetized, maintained, and catheterized to measure core temperature and cardiac output (CO) as described above. Blood samples were removed and hemodynamic parameters and arterial blood gases were recorded as above. Bioassays of chemokines from 1.0 ml samples of citrated plasma were conducted as previously described. Kajikawa et al., J. Immunol. Meth. 197:19-29, 1996; Johnson et al., J. Biol. Chem. 271:10853-10858, 1996, each incorporated herein by reference.

Sandwich enzyme-linked immunosorbent assays (ELISA) were constructed using goat polyclonal anti-recombinant rabbit IL-8, MCP-1, or GRO IgG as the both the capturing and detecting antibody. Anti-recombinant rabbit IL-8 and MCP-1 antibody was diluted to 200 mg/mL with 0.1 M bicarbonate buffer pH 9.6 and anti-recombinant rabbit GRO antibody was diluted 1:350 in the same buffer. Polystyrene 96-well plastic plates (Coster, Cambridge, MA) were coated with antibody solutions and incubated overnight at 4°C. The plates were rinsed twice with Dulbecco's PBS and blocked with 110% non-fat milk for 1 h at 37°C.

Samples (200 mL aliquot of citrated rabbit plasma diluted 1:4) and standards (serial dilutions of recombinant rabbit IL-8, MCP-1, or GRO protein) were added, and the plates were incubated for 2h (IL-8 and MCP-1) or 1 h (GRO) at 37°C. After washing 4 times, biotinylated immune goat IgG (1:800 dilution) was added to the plates and they were again incubated for 2h (IL-8 and MCP-1) or 1 h (GRO) at 37°C. After washing the plates were incubated with streptavidin biotin peroxidase complex reagent (Zymed, Inc., SanFrancisco, CA) at 37°C for 1 h. The peroxidase substrate (3,3',5,5'-tetramethylbenzidine, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added (100 mL/well) and incubated at 37°C for 1 h.

The reaction was stopped with 100 mL/well 1.0 M phosphoric acid and the optical density (450 nm) in each well was measured using a microtiter plate reader (Dynatech Co., Chantilly, VA). Cytokine concentration in the plasma samples was determined based on standard curves generated.

RESULTS

In the saline control rabbits of the IL-1α study group, no detectable levels of IL-8 (< 0.1 ng/mL), and only low levels of MCP-1 (< 0.45 ng/mL) and GRO (< 0.4 ng/mL) that were not significantly different at any time point, were observed. However, there was a sharp increase in IL-8 after IL-1a administration in both the untreated group and the group treated with PDTC, which increase was maximal at 120 minutes (Figures 16-18). The IL-8 response was even greater in the PDTC group than that observed in the IL-1a positive control group (1.12 ± 0.57 ng/mL vs 0.55 ± 0.28 ng/mL).

In contrast to PDTC, ProDTC treatment greatly attenuated this chemokine response (Figures 16-18). IL-8 levels rose to only 0.25 ± 0.15 ng/mL after 120 minutes. A similar pattern was observed for MCP-1 and GRO plasma levels. Significantly increased amounts of MCP-1 and GRO protein were detected in the plasma of rabbits treated with intravenous IL-1a when compared to rabbits treated with saline alone ($P < 0.05$). MCP-1 plasma levels peaked 120 minutes after IL-1a

infusion (17.77 ± 3.11 ng/mL) and remained significantly elevated for the duration of the study period. MCP-1 plasma levels were not altered in response to IL-1a in the PDTC treated group as the level of MCP-1 detected after 120 minutes was significantly elevated at 24.8 ± 4.03 ng/mL and remained significantly elevated for the entire study ($P < 0.05$).

Treatment with ProDTC completely prevented the IL-1α induced increase in MCP-1, with plasma levels reaching a maximum of only 7.73 ± 4.17 ng/mL after 120 minutes and remaining depressed throughout the study period (Figures 16-18). The plasma levels of MCP-1 were significantly lower in the ProDTC treated group at every time point compared to those measured in the PDTC treated group (Figure; $P < 0.05$).

The MCP-1 levels measured in the ProDTC treated group were not significantly different from those measured in the saline control group at any time point. IL-1a induced a rapid increase in the GRO chemokine which peaked at 60 minutes in the untreated group. GRO levels rose unabated in the PDTC treated group (Figures 16-18). Plasma levels of GRO were significantly elevated after IL-1a in the untreated and PDTC treated group at every time point compared to the saline control group ($P < 0.05$). ProDTC attenuated this response as GRO levels rose to only 3.37 ± 0.91 ng/mL after 120 minutes vs 5.45 ± 0.72 and 5.77 ± 0.75 ng/mL in the untreated positive control and PDTC treated groups respectively.

The plasma level of GRO measured in the PDTC treated group was significantly higher than that measured in the ProDTC group at 60, 120, and 180 minutes after IL-1a injection ($P < 0.05$). The plasma level of GRO in the ProDTC treated animals was not significantly different from that measured in the saline controls after 180 minutes (Figures 16-18).

As the above data indicate, IL-1α consistently caused a significant increase in the chemokines which peaked at about 120 minutes. ProDTC demonstrated an ability to abrogate this response while PDTC did not. In fact IL-8 and MCP-1 levels were greater in the PDTC treated animals than in the IL-1α controls. These results demonstrate that ProDTC

effectively attenuates cytokine induced elevation in chemokines observed during inflammatory states, thereby actively preventing adverse sequelae of such conditions attributable to chemokine stimulation.

PDTC on the other hand appears to be detrimental in the context of an acute inflammatory response, as this compound appears to act synergistically with IL-1α to elevate plasma chemokine levels. Such activity would be expected to exacerbate an uncontrolled, excessive inflammatory response.

The data provided herein indicate that, even though PDTC has been reported to inhibit IL-1-induced NF-κB nuclear binding activity necessary for expression of the chemokines (IL-8, MCP-1, GRO) *in vitro* (see, eg., Mukaida et al., J. Immunol. 143(4):1366-71, 1989; Siebenlist et al., Ann. Rev. Cell Biol. 10:405-455, 1994, each incorporated herein by reference), PDTC treatment actually increases the levels of these chemokines beyond levels seen in rabbits receiving only IL-1. In contrast, ProDTC treatment decreases chemokine expression in response to IL-1.

The mechanism underlying the adverse stimulation of chemokines in the presence of PDTC may relate to the severe acidosis induced by this compound. This response triggered by PDTC may overwhelm the reported effects of this compound of inhibiting NF-κB, thereby promoting a net deleterious response to IL-1. In contrast, ProDTC, which does not induce acidosis at comparable dosage with PDTC, effectively prohibits the chemokine response to IL-1.

An alternative explanation of this mechanism may relate to the requirement exhibit by chemokines for NF-κB activation to achieve their expression. In this context, PDTC is expected to be less effective in preventing cellular activation because it does not block NF-κB as completely and effectively as ProDTC. Our experiments indicate that ProDTC reliably and effectively blocks NF-κB.

The evidence herein indicates that PDTC has little or no effect on the inhibition of chemokine levels in response to cytokines, and that it may in fact exacerbate this

deleterious response. ProDTC on the other hand, effectively prevents the cytokine induced increase in plasma chemokines (IL-8, GRO, and MCP-1). This provides further evidence that PDTC acts via an alternative pathway or mechanism compared to ProDTC, or that ProDTC acts via pathways or mechanisms in addition to those it might share with PDTC.

While not wishing to be bound by theory, the unique ring structure of ProDTC and other cyclic DTC derivatives disclosed herein is thought to enable stereotypic inhibition of the IL-1 receptor complex or additional mediators or cytokine receptors induced by IL-1 at the cell surface. By this mechanism, inflammatory response to injury should be more effectively attenuated by ProDTC and other cyclic DTC derivatives compared to non-cyclic DTCs, as the present data demonstrate.

Another mechanism that may account for these distinct activities may involve an interaction between ProDTC and a G-protein or other second messenger that is unaffected by PDTC. Alternatively, ProDTC may block one of the several kinases or signalling proteins involved in the MAPK or SAPK pathways of activation that have been implicated in chemokine induction.

The results presented herein also raise the possibility that other transcription factors, in addition to NF-κB, are involved in the regulation of chemokines *in vivo*. In particular, these results indicate that NF-kB is not a sole regulator of cytokine-mediated gene expression *in vivo* involved in oxidative stress-induced inflammatory events. Thus, ProDTC may exhibit its effects on a transcription factor that is inaccessible or unaffected by PDTC.

In support of this model, Shreck and colleagues have shown that PDTC can inhibit LPS-, TNF-α-, and PMA-induced NF-kB activation, but had no affect on activation of the transcription factors, AP-1, octamer-binding proteins, cAMP response element binding proteins, or the transcription factor Sp-1, all of which have been implicated as regulators of

inflammation. Schreck et al. J. Exp. Med. 175(5):1181-94, 1992, incorporated herein by reference.

Although both PDTC and ProDTC exhibit antioxidant properties, not all antioxidants can inhibit the nuclear localization of NF-κB; suggesting a more specific mechanism of action for these compounds. Chelation properties may prevent transition metal-mediated free radical chain propagation and inhibit generation of free radicals by chelating heavy metals (eg., iron) utilized as cofactors by enzymes that stabilize changes in the intracellular redox potential (eg., superoxide dismutase). Addition of heavy metal ions in monocytes has been shown not to affect any inhibitory effects of PDTC, arguing against chelation as a central mechanism of action for this compound (ref 85 MT). Superior chelating properties of ProDTC may thus account for the ability of this compound to effectively prevent inflammatory responses, such as the chemokine response, that are mediated in part by intracellular transition metals acting in signalling, or as cofactors, for particular enzymes such as superoxide dismutase.

Further, it is possible that ProDTC, unlike PDTC, may inhibit transcription events mediated by zinc fingers. Additionally, PDTC has been shown to chelate extracellular metals, such as copper, and carry them across the cell membrane. Orrenius et al., Biochem. Soc. Trans. 24:1032-1038, 1996, incorporated herein by reference. Dissociation of displaced metals can lead to increased generation of reactive oxygen species, and thereby enhance oxidative damage reflected as an increase in chemokine production as the cell responds to injury. Thus, the more unstable the chelator the less able it is to prevent free radical production from transported metal ions. ProDTC and other cyclic DTC derivatives disclosed herein are more stable *in vivo*, and thus less likely than PDTC and other DTCs to cause an increase in free metal ions. ProDTC may have an affect on translation that is absent with PDTC. In addition to these possible mechanisms, ProDTC and its related derivatives exhibit greater stability and bioavailabilty than PDTC, which likely improves delivery of the drug and may

account in part for its ability to inhibit chemokine responses, in addition to their other, superior anti-inflammatory effects.

EXAMPLE V

DTCs Preferentially Inhibit Oxidative Stress-Induced NF-κB Activation

Although endothelial cell activation is known to be mediated through NF-κB in response to diverse stimuli, it has been heretofore uncertain whether each stimulus activates NF-κB through the same signalling pathway, or through different pathways or mechanisms. The existence of multiple pathways would make it possible to more selectively inhibit NF-κB activation in the setting of oxidative stress.

The possible existence of multiple pathways for NF-κB activation was explored by exposing human umbilical vein endothelial cells (HUVEC) to hypoxia, reoxygenation after hypoxia, or a reactive oxygen intermediate (H_2O_2). HUVEC were also treated with a specific tyrosine phosphatase inhibitor, pervanadate, to examine tyrosine phosphorylation of IkB in oxidative stress-mediated NF-κB activation. Different sample groups of HUVECs were exposed to medium alone or TNF-α, and either nuclear proteins were extracted for electrophoretic mobility shift assays (EMSAs) to determine NF-κB activation, or cytoplasmic proteins were extracted for Western blotting to determine the presence or absence of IκBα.

Execution of these experiments, described in further detail below, yielded surprising evidence that there is, in fact, an alternative pathway or mechanism for NF-κB activation. This pathway or mechanism is triggered specifically by conditions of oxidative stress, and is specifically blocked by cyclic DTC derivatives disclosed herein.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase (Worthington Biochemical, Freehold, NJ) digestion. Cultures were maintained in RPMI 1640 (Whittaker, Walkerville, MD) supplemented with 10% adult bovine serum (JRH Biosciences, Lenexa, KS), heparin (90 units/mL; Sigma, St.

Louis, MO), endothelial cell growth supplement (50 units/mL; Collaborative Research, Bedford, MA) and antibiotics in room air at 37°C. HUVEC were serially passaged by brief exposure to Versene (GIBCO/BRL, Grand Island, NY) and 0.05% Trypsin with EDTA (GIBCO/BRL, Grand Island, NY). Third passage confluent HUVEC monolayers were used in all experiments.

Hypoxia and Reoxygenation

Normoxic control cells were maintained in a standard incubator at room air oxygen tension (21% oxygen) at 37°C designated "normoxia". To expose HUVECs to hypoxia, cultures were placed in a controlled environmental chamber (Coy Laboratory Products, Ann Arbor, MI) at 37°C and maintained at oxygen tension of 2-3% oxygen. Oxygen content in the media over the HUVECs equilibrated with the hypoxic environment of the chamber within 10 minutes as measured by a Clark electrode (data not shown). To expose HUVECs to hypoxia and reoxygenation, cultures were removed from the hypoxia chamber and placed in the normoxic incubator for designated periods of time.

Electrophoretic Gel Mobility Shift Assays

Third passage HUVEC monolayers grown in 100 mm^2 plates were exposed to conditions of oxidative stress (hypoxia/reoxygentation, 500μM H_2O_2, or 200μM pervandate) or TNF-α (200 units/ml) for designated time periods. Nuclear protein extractions were performed according to conventional methods (see, eg., Deryckere et al, Biotechniques 16:405, 1994, incorporated herein by reference). Approximately 20 μg of nuclear protein were incubated in a binding reaction with a ^{32}P-end-labeled oligonucleotide containing a human consensus NF-κB binding sequence (Promega) (see, eg., Schreck et al. J. Exp. Med. 175(5):1181-94, 1992, incorporated herein by reference). Binding reactions occurred at room temperature for 20 minutes. Proteins were resolved on a 6% nondenaturing polyacrylamide gel at 100 V for 1-2 hours in a 0.5% TBE buffered solution. The gels were dried and blotted overnight, and autoradiographed.

West rn Blot Analysis: Confluent HUVEC monlayers on 100mm^2 plates were treated with conditions of oxidative stress

as described above, or TNF-α. Cytoplasmic extracts were obtained using a modified lysis buffer, and total protein concentration was determined using a standard protein assay. Approximately 20 μg of protein were loaded on PAGE-SDS gels and run at 100 volts for two hours. Following transfer to a PVDF membrane, the membrane was stained with Coomasie blue to determine equal protein transfer. The membranes were then incubated with anti-IκBα polyclonal antibody (Santa Cruz Biotechnonlogy) at 1:1000 dilution for 2 hours. An HRP conjugated secondary antibody was applied for 1 hour, and the proteins were visualized using Amersham ECL reagents and autoradiography.

RESULTS

Referring to Figures 19-21, TNF-α induced rapid degradation of IκBα protein, with concomitant nuclear translocation of NF-κB within 5-10 minutes. Complete IκBα degradation was observed after 15 minutes, with restoration of IκBα by 1 hr during continuous exposure to TNF-α. Similar results were obtained when cells were treated with IL-1β or LPS.

HUVEC cultures that were exposed to 2 hours of hypoxia followed by reoxygenation demonstrated rapid nuclear translocation of NF-κB within 15 minutes, similar to that observed when HUVEC were treated with TNF-α (Figure 19). Rapid nuclear translocation of NF-κB also occurred in HUVECs exposed to hypoxia alone, or treated with H_2O_2 (Figures 19 and 20). However, in contrast to the treatment of cells with TNF-α, reoxygenation of hypoxic HUVEC did not result in degradation of IκBα (Figure 19). Similarly, exposure of HUVEC to hypoxia, or treatment of HUVEC with H_2O_2, induced a similar pattern of NF-κB nuclear translocation without IκBα degradation (Figures 19 and 20).

Pervanadate consists of H_2O_2 and vanadate, a tyrosine phosphatase inhibitor. Pervanadate thus preserves transient tyrosine phosphorylation of proteins and potentiates signal transduction pathways activated by H_2O_2 (or other ROIs).

Moreover, tyrosine phosphorylation of IκBα in cells treated with pervanadate is reported to block IκBα degradation. As shown in Figure 20 pervanadate treatment initiated rapid and pronounced nuclear translocation of NF-κB without associated degradation of IκBα. Neither IκBα nor IκBε underwent degradation in HUVEC exposed to oxidative stress.

The above findings indicate that oxidative stress activates NF-κB through an alternate pathway that does not involve IκBα degradation required for NF-κB activation induced by all other stimuli known to activate this transcription factor. Furthermore, IκBβ or IκBη degradation, as measured by Western blot analysis with polyclonal antibodies specific for each inhibitor protein were not observed in HUVECs exposed to oxidative stress.

The degradation of IκB is surmised to be an essential step during NF-κB activation. Singh et al., J. Biol. Chem. 271 (49):31049-54, 1996, incorporated herein by reference. TNF-α, LPS, and PMA stimulation of HUVEC results in rapid serine phosphorylation of IκBα, followed by ubiquination and degradation. Brown et al., Science 267 (5203):1485-8, 1995, incorporated herein by reference. In addition, recent reports have demonstrated that IkB can be phosphorylated on a tyrosine residue at position 42 of IκB, in close proximity to the two serine phosphoacceptor sites. Tyrosine phosphorylated IκB is protected from TNF-induced degradation, although the mechanism of this protective effect is not known.

Koong, however, described tyrosine phosphorylation of IkBα following exposure to hypoxia in Jurkat T cells. In contrast to our findings, he also showed evidence of IκBα degradation at 60 minutes hypoxia. Koong et al., Cancer Res. 54:5273-5279, 1994, incorporated herein by reference. Imbert et al, also using Jurkat T cells, subsequently described tyrosine phosphorylation of IκBα following exposure to pervanadate and hypoxia reoxygenation, both forms of oxidative stress. In addition, their study reported that tyrosine phophorylation leads to dissociation of IκBα from NF-κB rather

than degradation induced by cytokine exposure. Imbert et al., Cell 86(5): 787-98, 1996, incorporated herein by reference.

Based on the foregoing evidence, it is possible that oxidative stress activates a signaling pathway resulting in tyrosine phosphorylation of IκBα, rather than serine phosphorylation induced by TNF. Tyrosine phosphorylation causes IκBα to dissociate from NF-κB but, unlike serine phosporylation, does not signal IκBα degradation.

The examples and data presented herein demonstrate that oxidative stress activates the proinflammatory transcription factor NF-κB through an alternative pathway or mechanism from previously described pathways. This discovery allows targeting and blockade of the proinflammatory transcriptional response to oxidative stress (eg., as occurs in I/R), while leaving the endothelial cell's ability to respond to infection, or to beneficial cytokine signals, intact.

The results thus provide novel methods using cyclic DTC and TS derivatives to target the aforementioned, specific pathway or mechanism for oxidative stress-induced NF-κB activation. Although there is considerable evidence demonstrating that stimuli associated with septic challenges, as induced by TNF and LPS, initiate signaling pathways that lead to NF-κB activation through serine phosphorylation and then degradation of IkBα by the proteasome, little has been previously known about the signaling events leading to NF-κB activation in response to oxidative stress.

The foregoing examples and data suggest that a central difference between oxidative stress and infectious stimuli is attributable to a fundamental difference in IkBα phosphorylation. Contrary to the behavior of other inflammatory pathways, in the discrete setting of oxidative stress IkBα phosphorylation does not result in IkBα or IkBβ degradation. These and related findings indicate that oxidative stress activates NF-κB through an independent and distinct signal transduction pathway leading to the tyrosine phosphorylation of IkBα.

To further define oxidative stress-induced inflammatory mechanisms, the ability of a cyclic DTC derivative, ProDTC, to inhibit oxidative stress-induced NF-κB activation was examined herein. These studies indicate that Pro-DTC blocks oxidative stress-induced NF-κB activation. However, when the ability of ProDTC to inhibit TNF-induced NF-κB activation was tested, no substantial effect was observed.

Non-cyclic DTCs, as exemplified by PDTC, are reported in the literature to inhibit TNF-induced NF-κB activation. Satriano et al., J. Clin. Invest. 94(4):1629-36, 1994, incorporated herein by reference. This report, combined with the data herein, further indicates that the cyclic DTC and TS derivatives used within the present invention, exemplified by Pro-DTC, selectively inhibits an oxidant pathway for NF-κB activation.

The selectivity of ProDTC and its related compounds for inhibiting an oxidant pathway involved in NF-κB activation may be due to a specific affinity that these compounds have for the kinase that tyrosine phosphorylates NF-κB under oxidative stress. Because oxidative stress and infectious stimuli (eg., TNF and LPS) appear to signal NF-κB activation through different pathways, and because the cyclic DTC and TS derivatives described herein selectively inhibit this pathway, these compounds allow specific blockade of ischemia-reperfusion or oxidative injury, without effecting the host's ability to respond to infectious stimuli. This discovery provides a major advantage over currently available techniques to prevent microvascular activation, such as the use of monoclonal antibodies to prevent neutrophil adhesion, which are known to greatly increase the host risk of infection.

EXAMPLE VI

Cyclic DTC Derivatives Prevent Myocardial Ischemia-Reperfusion Injury

Ischemia-reperfusion (I/R) injury exists as a continuum ranging from mild stunning, characterized by reversible post-ischemic organ dysfunction, to infarction,

which is characterized by irreversible myocellular necrosis and programmed cell death (apoptosis). Boyle et al., Ann. Thorac. Surg. 62(6):1868-75, 1996; Lefer et al., Ann. Rev. Pharmacol. Toxicol. 33:71-90, 1993, each incorporated herein by reference. In many patients, especially those who have profound coronary ischemia, reperfusion of ischemic myocardium can lead to severe cardiogenic shock, with resulting multiorgan dysfunction, and even death.

Recently Rupec and Baeurle demonstrated in Jurkett T cells that, under strict conditions of hypoxia, AP-1 alone is activated, and when hypoxic cells are reoxygenated NF-κB is activated. Rupec et al., Eur. J. Biochem. 234(2):632-40, 1995, incorporated herein by reference.

This evidence indicates that reperfusion is a significant event in NF-κB activation. It is unknown, however, what role NF-κB plays in regional myocardial ischemia-reperfusion injury *in vivo*. In this example, the ability of cyclic DTC or TS derivatives that inhibit phosphorylation of IkB-α and the release of NF-κB to the nucleus to inhibit regional myocardial ischemia-reperfuison injury is demonstrated.

In Situ Coronary Ligation Model

To evaluate the effect of inhibiting NF-κB on injury sustained during ischemia-reperfusion, we utilized a well-characterized regional ischemia-reperfusion injury model. In this model, rabbits were housed, anesthetized, maintained, and catheterized to measure core temperature and cardiac output (CO) as described above.

To achieve coronary ligation, the heart was exposed via median sternotomy. A 5F Millar catheter was placed through a small pursed-stringed incision in the left ventricle to allow estimation of the left ventricular peak systolic pressure (LVPSP) and left ventricular end diastolic pressure (LVEDP). A 4.0 Vicryl suture was passed twice around a large arteriolateral branch of the left main coronary artery supplying the majority of the left ventricle, and the ends of the suture passed through a small length of polyethene tubing to form a snare.

After a 20-30 minute stabilization period, baseline values were recorded. Regional myocardial ischemia was produced by reversibly occluding the artery for 45 minutes followed by 120 minutes of reperfusion. A Millar catheter placed in the left ventricle to measure left ventricular end diastolic pressure, left ventricular end systolic pressure, ejection fraction, and the rise in left ventricular pressure (dP/dt). Sonometric piezoelectric crystals (Sonometrics, Inc, New London, Ontario) placed on the surface of the left ventricle to assess segmental contractility. The hemodynamic trace acquired from the Millar catheter allows segmental shortening measurements to be correlated with end diastolic and end systolic time points.

Study Groups

Six animals, referred to as group I, were given 1 cc of PBS 15 minutes prior to the I/R protocol. A second group of six animals, group II, were given 15 mg/kg PDTC dissolved in 1 cc of PBS intravenously before the I/R procedure. A third group of six rabbits, group III, was given 15 mg/kg of ProDTC intravenously dissolved in 1 cc of PBS before the procedure.

Determination of Infarct Size

At the completion of the 120-minute reperfusion period, the coronary artery was reoccluded, and 6 mL of 10% Evan's Blue dye (Sigma, Saint Louis, MO) was injected into the left atrium and allowed to circulate and stain all perfused tissue. The area supplied by the ligated vessel remained unstained, demarcating the myocardium at risk for infarction. After the heart was arrested with pentobarbital it was rapidly excised. The left ventricle was isolated from the rest of the heart, weighted, and then cut into 2 mm-thick transverse slices. The normal myocardium, which stained blue, was separated from the area at risk, which was unstained. The area at risk was placed in a 37°C solution of 1% triphenyl-tetrazolium chloride (TTC) for 30 minutes.

TTC stains the viable tissue brick red and leaves the necrotic zone pale. The TTC-red (non-infarcted) tissue was separated from the TTC-pale (necrotic) tissue and each area was

weighed. The left ventricular area at risk (LVAAR) was calculated as the sum of the noninfarcted and necrotic tissue weight of the tissue perfused by the occluded vessel divided by the weight of the left ventricle, expressed as a percent. The left ventricular area of necrosis (LVAN) was calculated as the weight of necrotic tissue divided by the weight of the left ventricle, expressed as a percent. The infarct size was calculated by dividing the weight of the TTC-pale tissue by the weight of the total area at risk (LVAN/LVAAR).

RESULTS

By TTC staining, 45% of the left ventricle of group I animals was at risk for infarction, of which 56% was infarcted (Figure 22). Group II animals (given 15 mg/kg ProDTC) had 44% of the left ventricle was at risk for infarction, of which 23% was infarcted. Comparatively, in group III rabbits (given 15 mg/kg of ProDTC), forty-five percent of the left ventricle was at risk for infarction, of which only 14% was infarcted (Figure 22). Thus, ProDTC administration results in a significantly improved reduction in myocardial infarct size in comparison to PDTC.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for inhibiting an inflammatory condition or disease in a mammalian patient comprising administering to the patient an antiinflammatory effective amount of a cyclic DTC or TS derivative.

2. The method of claim 1, wherein said patient suffers from or is susceptible to ischemia-reperfusion injury.

3. The method of claim 1, wherein said patient suffers from or is susceptible to systemic inflammatory induced shock.

4. The method of claim 1, wherein said patient suffers from or is susceptible to complications from cardiopulmonary bypass or extracorporeal membrane oxygenation (ECMO).

5. The method of claim 1, wherein said patient suffers from or is susceptible to an inflammatory condition or disease selected from intimal hyperplasia, atherosclerosis, arthritis, chronic inflammatory autoimmune disease, inflammatory skin disease, periodontal disease, pulmonary injury, and gastrointestinal inflammation.

6. The method of claim 1, wherein said patient suffers from or is susceptible to an adverse inflammatory condition associated with a surgical procedure or injury.

7. The method of claim 1, wherein said patient suffers from or is susceptible to infertility, heart attack, cancer, complications from diabetes, complications of chemotherapy, or complications of acquired immune deficiency syndrome.

8. The method of claim 1, wherein said cyclic DTC derivative has the general formula R1,R2-N-C(S)S-R3, wherein R1 and R2 constitute a cycle including a nitrogen atom of a dithiocarbamate.

9. The method of claim 1, wherein said cyclic TS derivative has the general formula R1,R2-N-C(S)-Sx-C(S)-N-R3,R4, wherein x is 1 to 8, and wherein R1, R2 and R3, R4 constitute independently a cycle including the nitrogen atoms of the respective dithiocarbamate residue.

10. The method of claim 1, wherein said cyclic DTC or TS derivative has at least one, 5- to 7-membered ring structure.

11. The method of claim 10, wherein said cyclic DTC or TS derivative has one or two substitutents in a bridge portion of said ring.

12. The method of claim 11, wherein said one or more substituents are selected from carboxyl, carboxylester, hydroxyl, nitro, amino or mercapto substitutents.

13. The method of claim 10, wherein said cyclic DTC or TS derivative has one or more heteroatoms selected from sulfur, nitrogen, or oxygen in a bridge portion of said ring.

14. The method of claim 10, wherein said cyclic DTC or TS derivative has one or two carbonyl functions.

15. The method of claim 10, wherein said cyclic DTC or TS derivative is dehydrogenated to yield one or two double bonds within said ring structure.

16. The method of claim 10, wherein said cyclic DTC or TS derivative has one or more structural characteristics selected from substituent(s) in a bridge portion of said ring,

heteroatom(s) within said bridge portion, carbonyl function(s), and/or dehydrogenation to yield one or two double bonds.

17. The method of claim 16, wherein said cyclic DTC or TS derivative is more hydrophilic as compared to hydrophilicity of a parent compound lacking said substituent(s), heteroatom(s), carbonyl function(s), and/or dehydrogenation.

18. The method of claim 1, wherein said cyclic DTC or TS derivative is selected from prolinedithiocarbamate (ProDTC), thioproline-dithiocarbamate (TProDTC), prolinethiuramidisulfide (ProTDS), or thioprolinethiuramidisulfide (TProTDS).

19. A method for inhibiting an inflammatory condition or disease in a mammalian patient comprising administering to the patient an antiinflammatory effective amount of a dithicarboxylate compound which does not substantially increase a blood pH level in said patient.

20. A method for inhibiting an inflammatory condition or disease in a mammalian patient comprising administering to the patient an antiinflammatory effective amount of a dithicarboxylate compound which does not substantially increase a blood pH level of said patient.

21. A method for inhibiting an inflammatory condition or disease in a mammalian patient comprising administering to the patient an antiinflammatory effective amount of a dithicarboxylate compound which does not substantially increase a level of an inflammatory-stimulating chemokine selected from IL-8, GRO and MCP-1 in said patient.

22. A method for inhibiting an inflammatory condition or disease in a mammalian patient comprising administering to the patient an antiinflammatory effective

amount of a dithicarboxylate compound which inhibits an activity of NF-κB but does not result in substantial degradation of IGkBα.

FIG. 1A

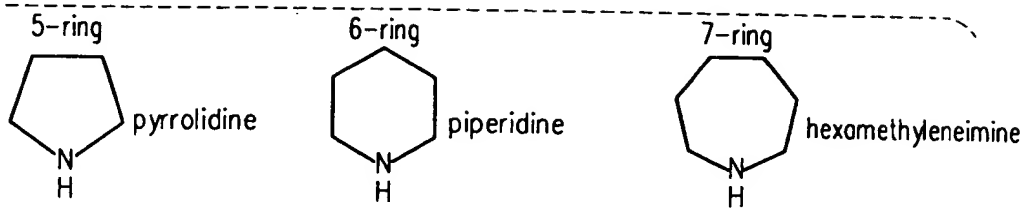


FIG. 1B

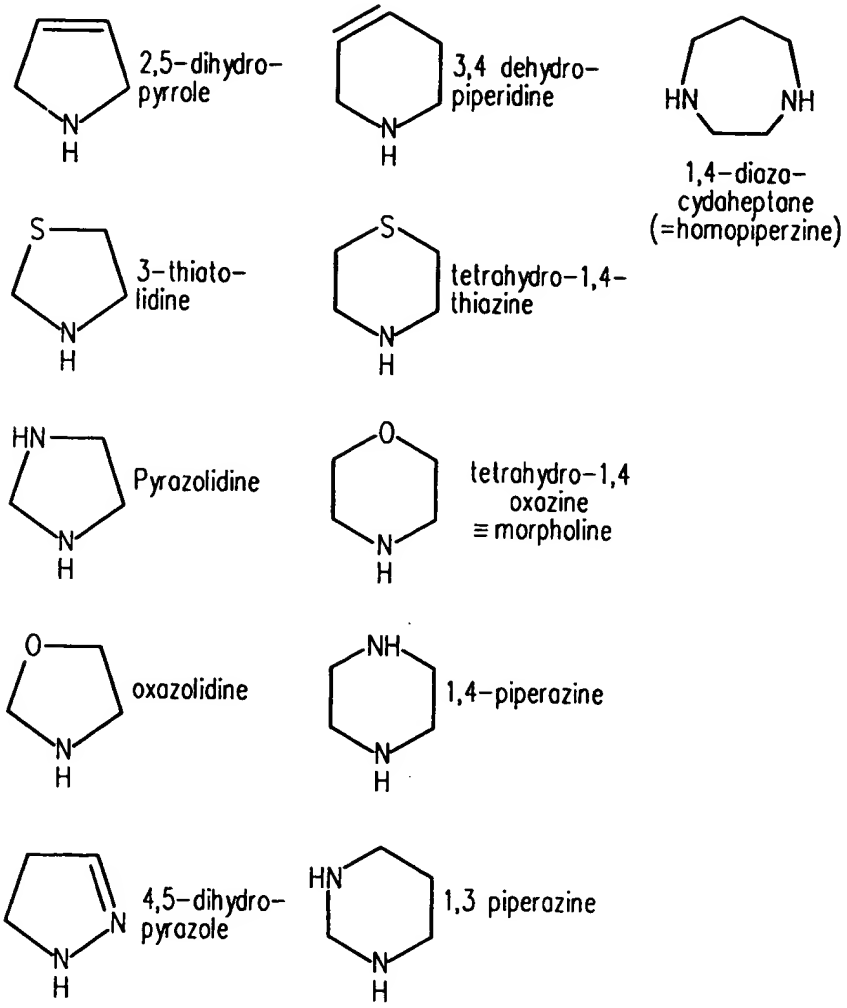


FIG. 2

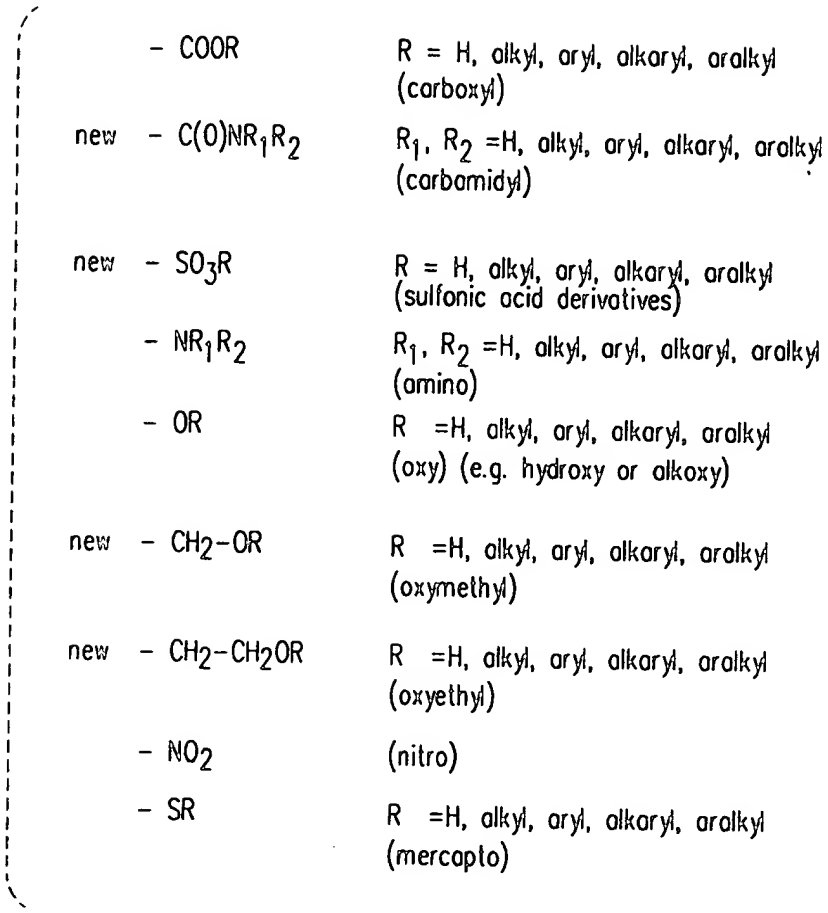


FIG. 3

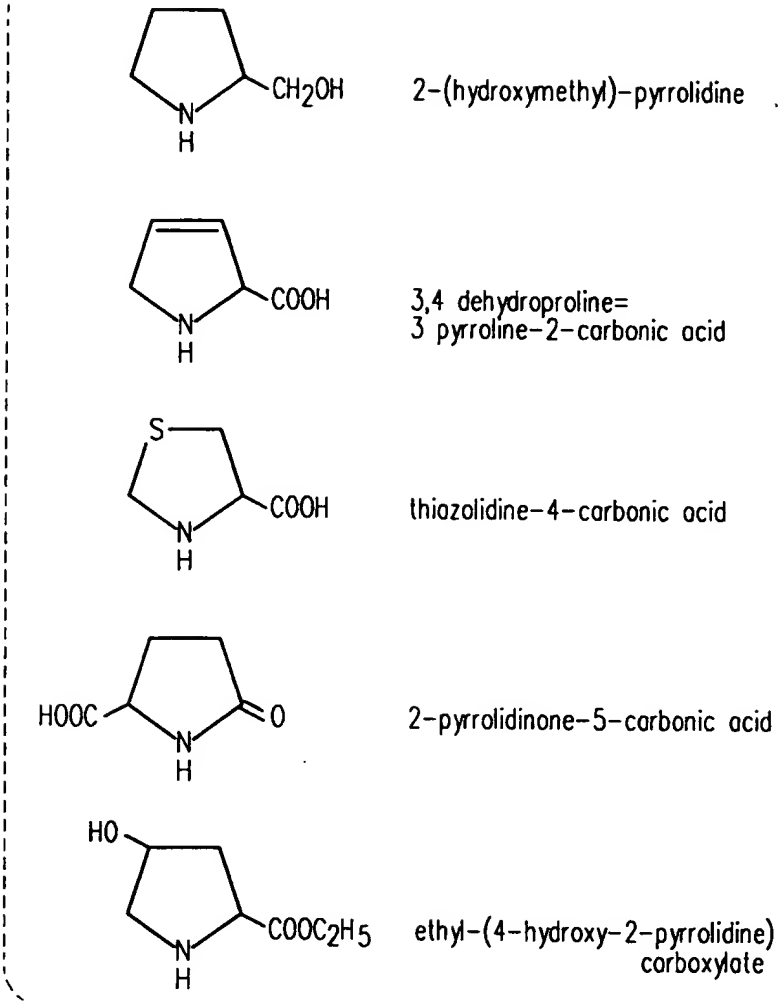


FIG. 4

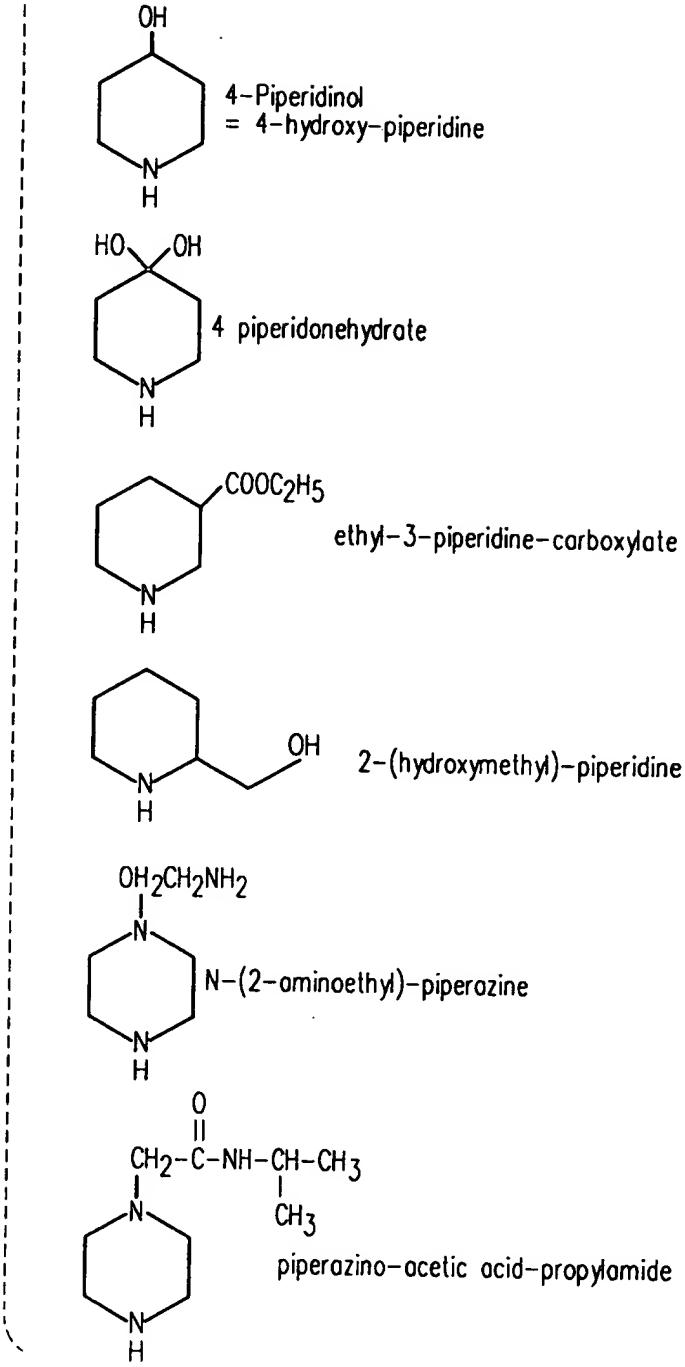
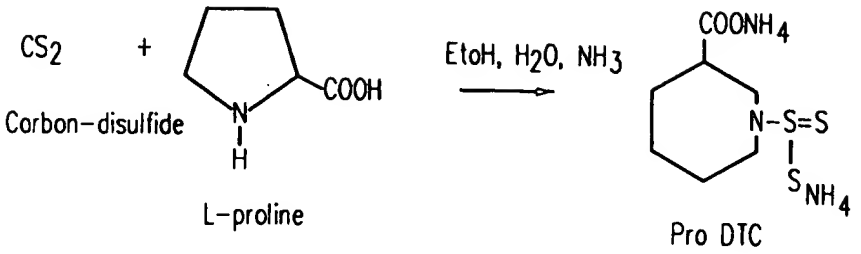


FIG. 5



also other bases could be used, yielding other salts.
(e.g. NaOH would give di-sodium salt)

Proposed mechanism:

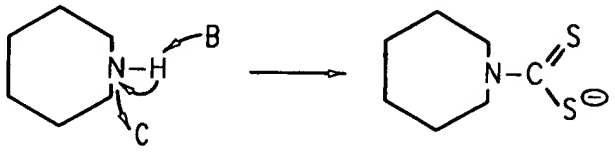
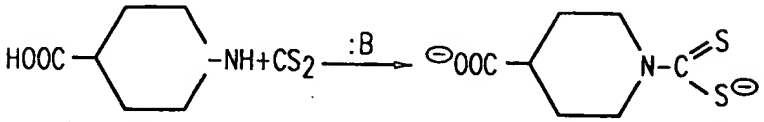


FIG. 6

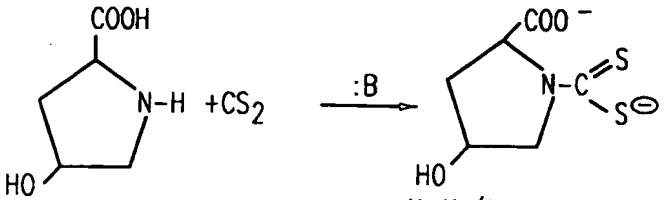
- Piperidine-4-carbonic acid



(we synthesized)

N, N-(3-carboxy-pentamethylene dithiocarbamate

- 4-Hydroxy-trans-proline



N, N-(1-carboxy-3-hydroxy)-tetra methylen-dithiocarbonate

FIG. 7

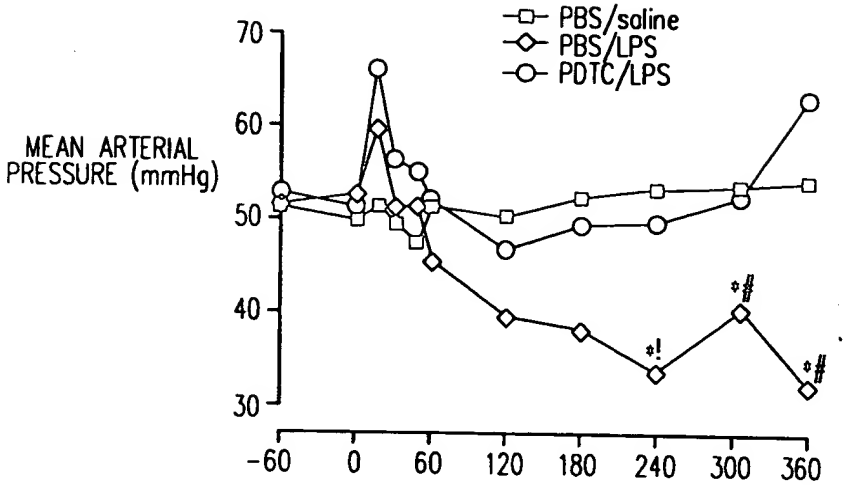


FIG. 8

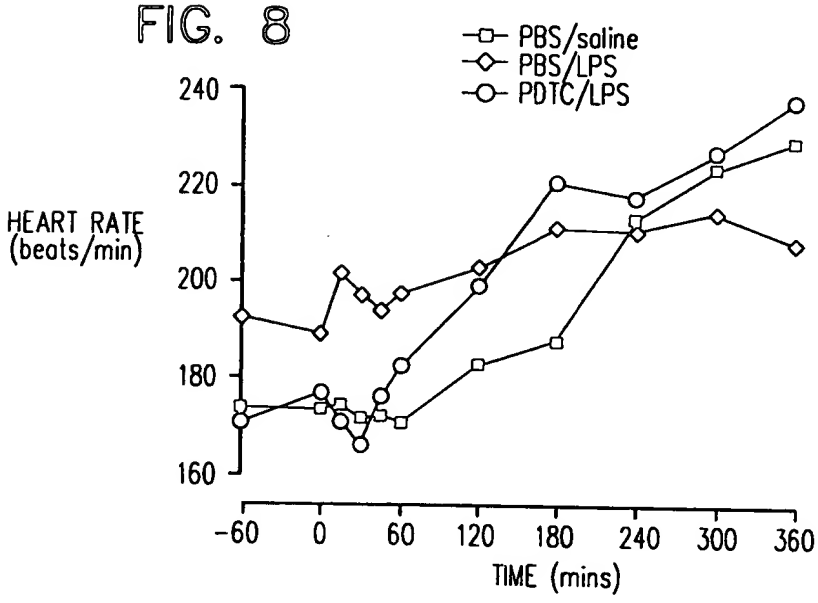


FIG. 9

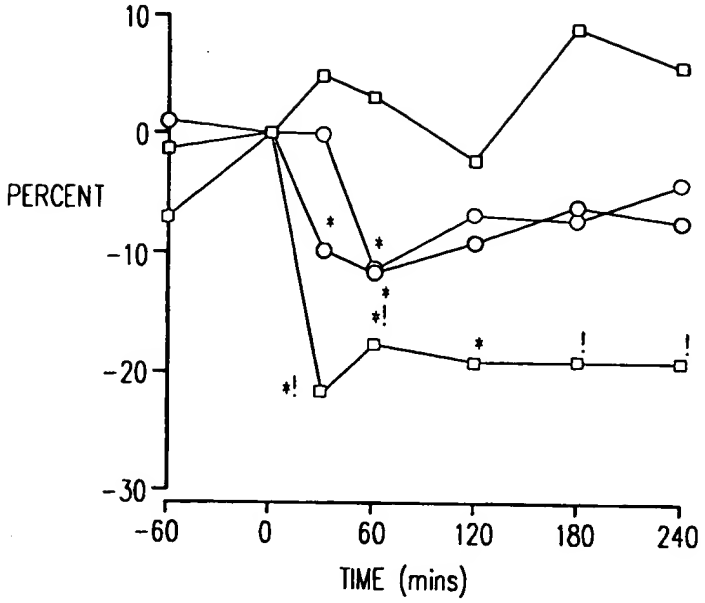


FIG. 10

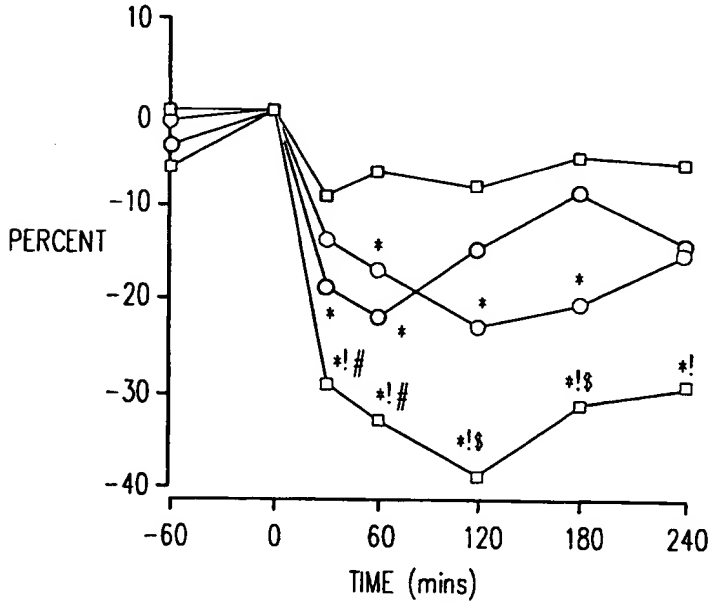


FIG. 11

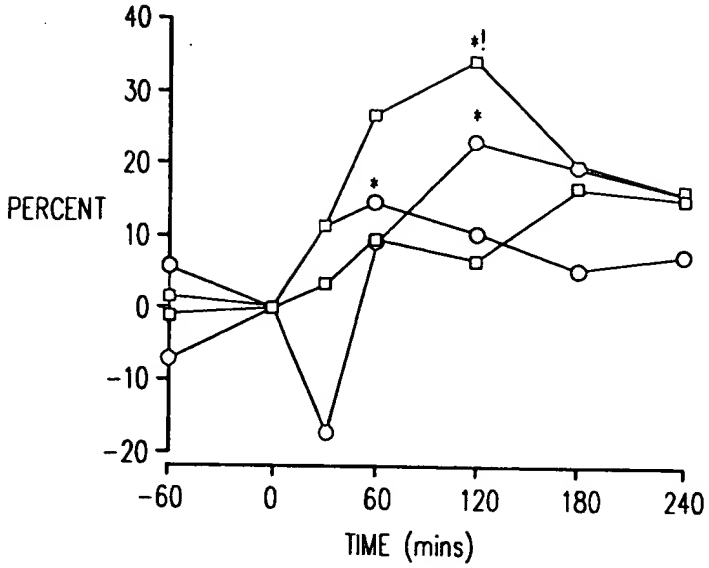


FIG. 12

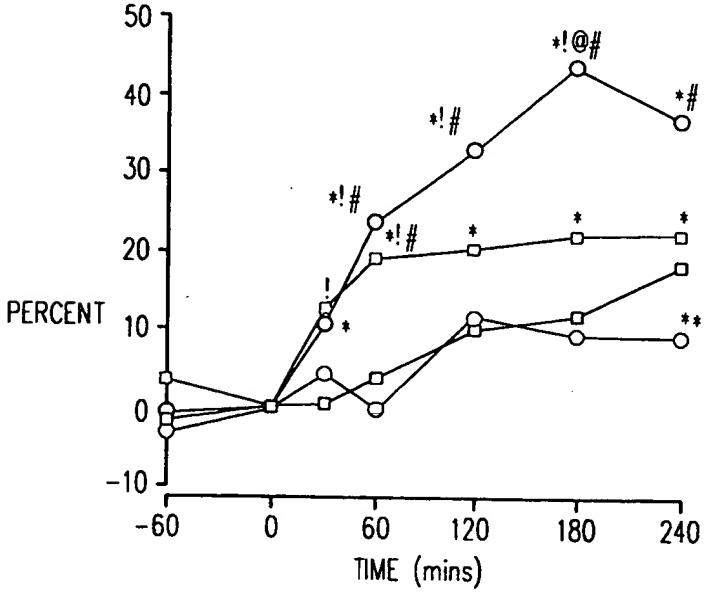


FIG. 13

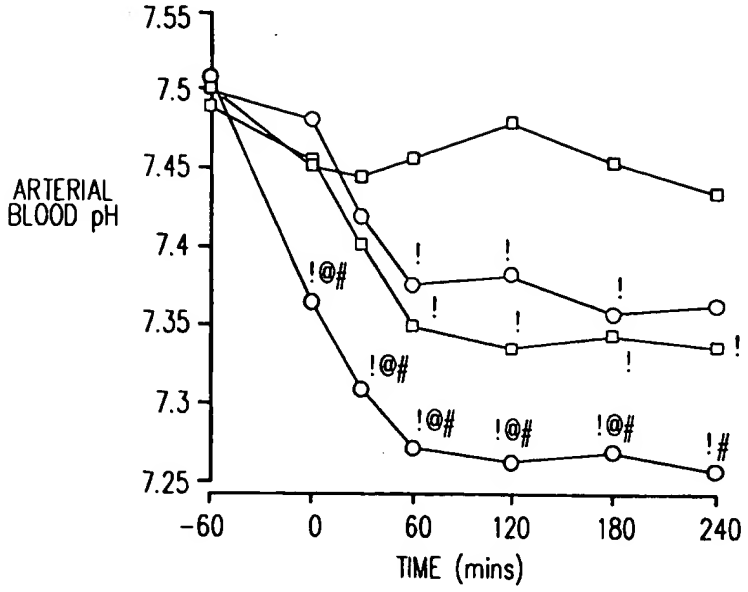


FIG. 14

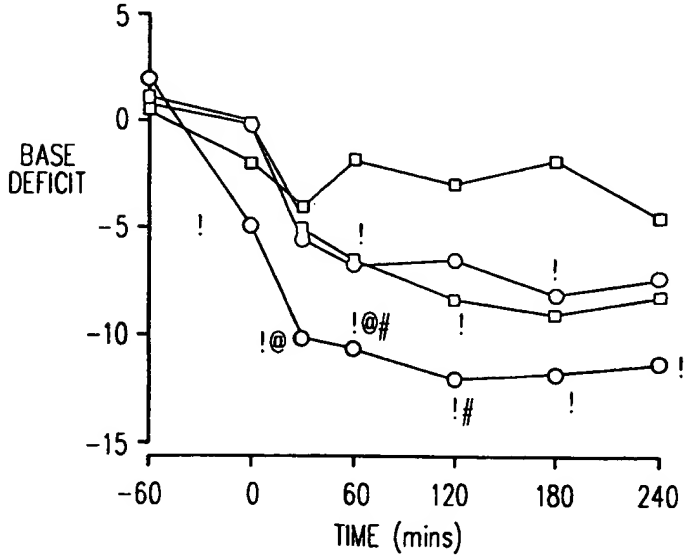


FIG. 15

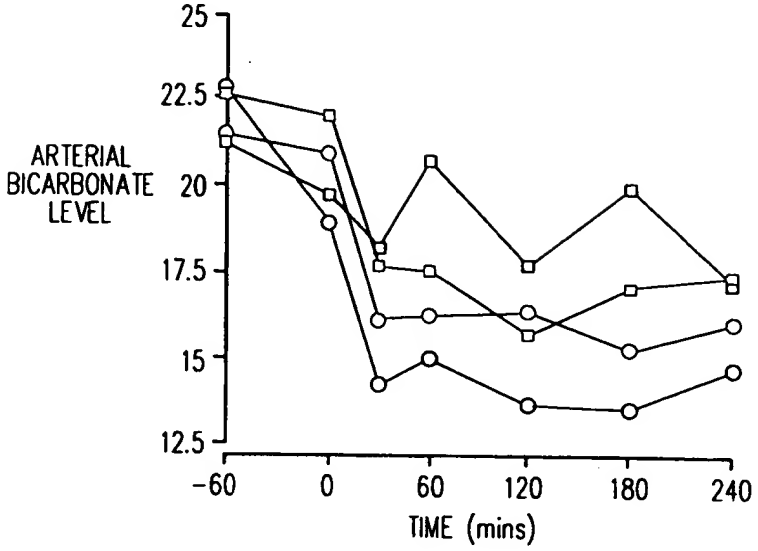


FIG. 16

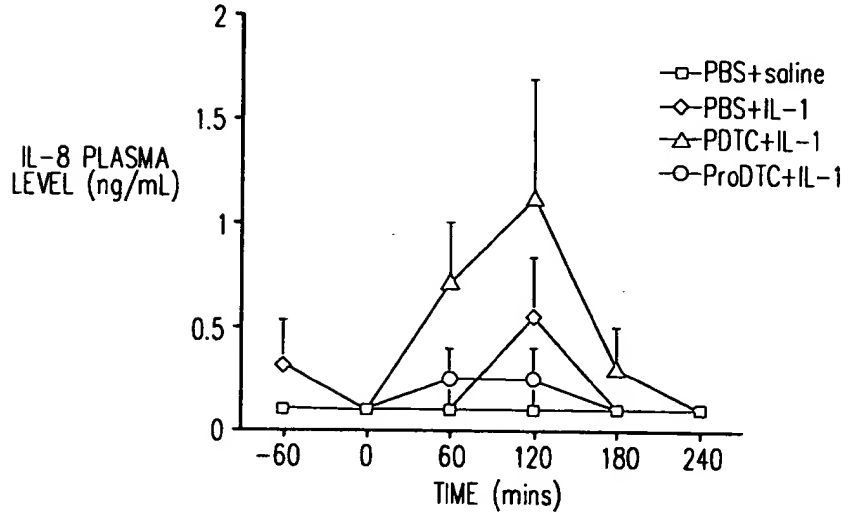


FIG. 17

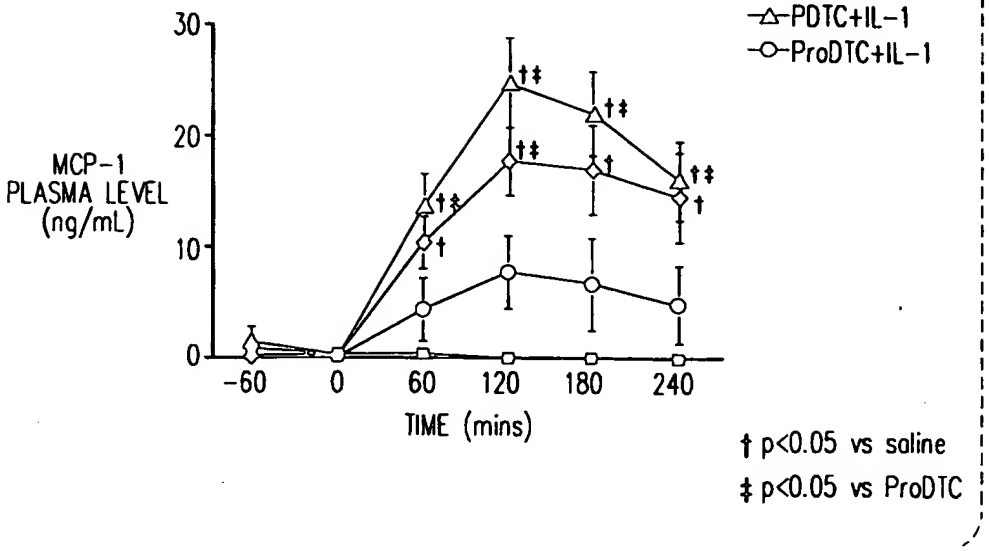


FIG. 18

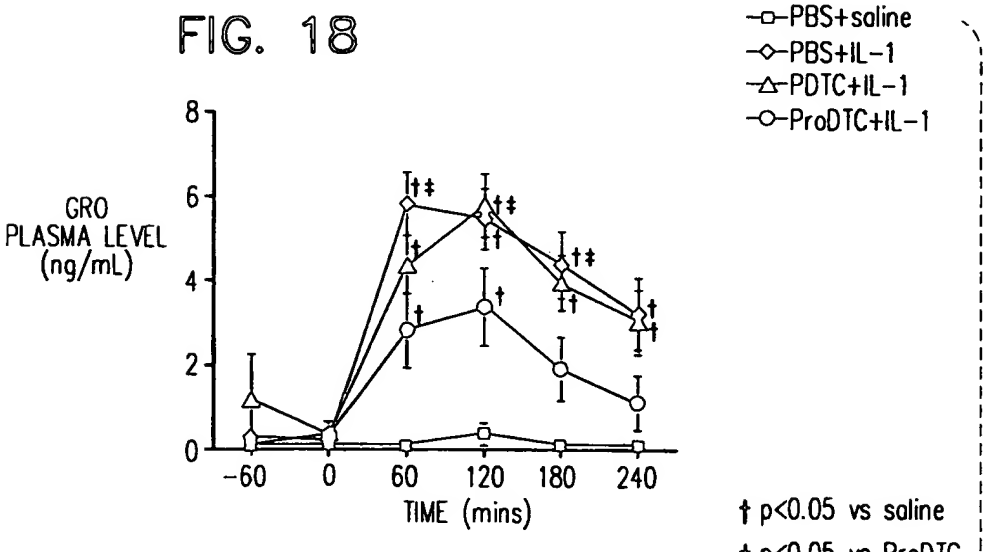


FIG. 19A

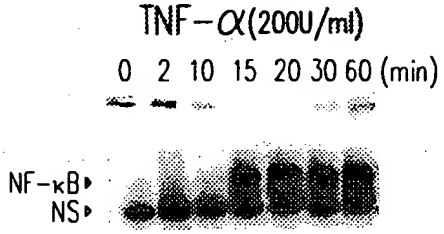


FIG. 19B

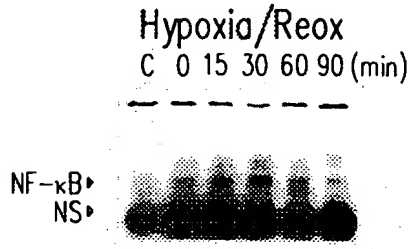


FIG. 20A

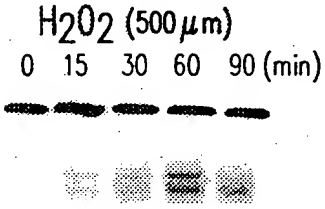


FIG. 20B

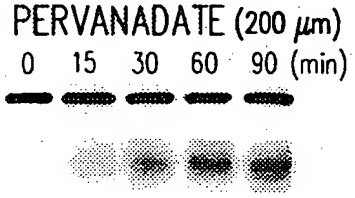


FIG. 21A

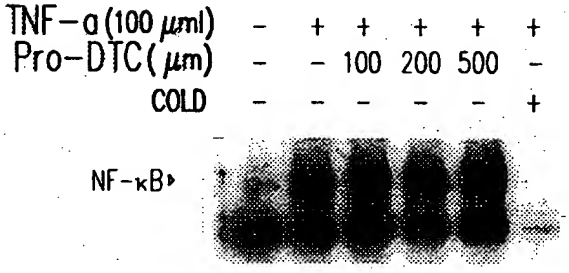


FIG. 21B

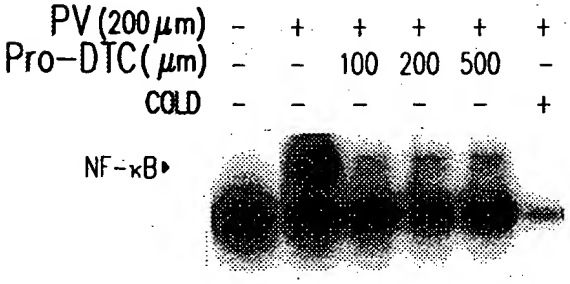
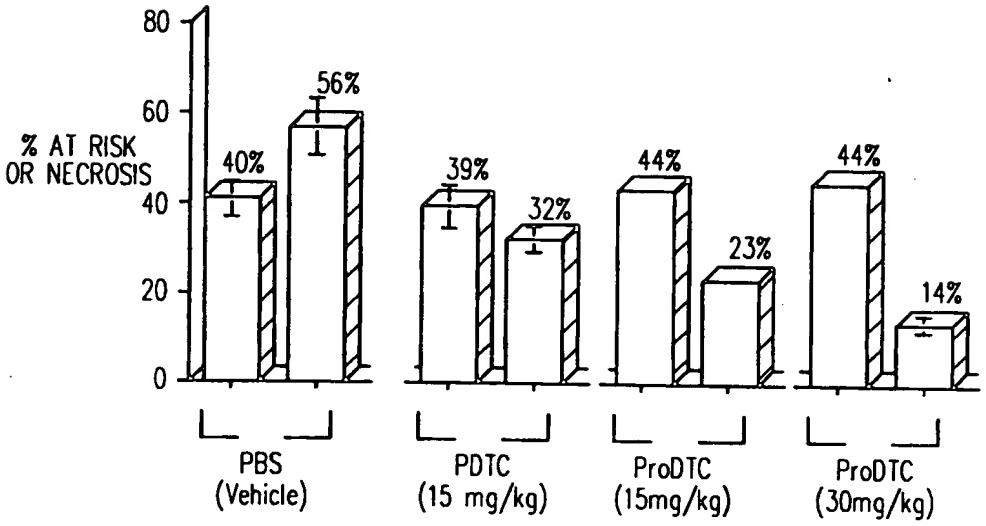


FIG. 22



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14490

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/40, 31/395, 31/55, 31/445
US CL :514/423, 210, 212, 315
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/423, 210, 212, 315

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,380,747 A (MEDFORD et al) 10 January 1995, col. 1-11 and the claims.	1-22
X,P	US 5,821,260 A (MEDFORD et al) 13 October 1998, col. 1-14 and the claims.	1-22
X	Database Chemical Abstracts on STN, American Chemical Society, No. 125:76341, HENDERSON, et al. 'A method for identifying and using compounds that inactivate HIV-1 and other retroviruses by attacking highly conserved zinc fingers in the viral nucleocapsid protein,' abstract, WO 9609406, 28 March 1996.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 AUGUST 1999	Date of mailing of the international search report 22 OCT 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer WILLIAM JARVIS JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)☆

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/14490

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Chemical Abstracts on STN, No. 125:184901, RICE, W. G. et al. 'Evaluation of Selected Chemotypes in Coupled Cellular and Molecular Target-Based Screens Identifies Novel HIV-1 Zinc Finger Inhibitors,' abstract, J. Med. Chem. 1996, 39(19), 3606-3616.	1-22